Acute Post-Ischemic Seizures are Associated with Increased Mortality and Brain Damage Following Hypoxia-Ischemia in Adult Mice

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Post-stroke seizures are associated with worsened outcome following stroke, but the underlying pathophysiology is poorly understood. Here I combined behavioral, electrophysiological and histological assessments to examine acute seizures in adult mice following hypoxia-ischemia (HI). C57BL/6 mice aged 4-9 months received a permanent occlusion of the right common carotid artery and were then exposed to systemic hypoxia (8% O₂, ~30 minutes). The HI episode resulted in decreases in cerebral blood flow, suppression of EEG activities and extensive brain injury in the hemisphere ipsilateral to the carotid artery occlusion. Generalized motor seizures were observed within 72 hours following HI. These seizures occurred nearly exclusively in animals with the extensive ipsilateral brain injury, but their generation was not associated with EEG discharges in bilateral hippocampal and cortical areas. Animals exhibiting these seizures had a high rate of mortality. Post-HI treatments with diazepam and phenytoin suppressed these motor seizures and improved post-HI animal survival. Based on these data, I conclude that these seizures are a consequence of HI brain injury, contribute to mortality following HI, and that they may originate from deep subcortical structures.
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Contents

Acknowledgments.................................................................................................................. iii

Contents .................................................................................................................................. iv

List of Figures ......................................................................................................................... ix

List of Appendices .................................................................................................................. xi

Chapter 1 ..................................................................................................................................1

1 Introduction ...........................................................................................................................1

1.1 A primer of stroke ..........................................................................................................1

1.2 Elementary principles of seizures ..................................................................................4

1.2.1 Towards a mechanistic definition of seizures and epilepsy ........................................5

1.2.2 How do seizures arise? ..............................................................................................6

1.2.3 The electroencephalogram and seizures ....................................................................9

1.2.4 A note on seizure classification ................................................................................10

1.2.5 Seizures summarized ................................................................................................12

1.3 General introduction to post-stroke seizures .................................................................12

1.4 Early-onset post-stroke seizures in humans ....................................................................14

1.5 Early-onset post-stroke seizures in rodents ..................................................................16

1.5.1 Generalized non-convulsive early-onset post-ischemic seizures ................................19

1.5.2 Generalized convulsive early-onset post-ischemic seizures ......................................20

1.5.3 Early-onset seizures in other models of cerebral ischemia ........................................21

1.6 Possible mechanisms of early post-stroke seizures .......................................................21

1.7 Post-ischemic seizures and brain injury ..........................................................................24

1.8 Generalized early-onset post-stroke seizures summarized ...........................................25
Chapter 2

2 Experimental model and hypotheses

2.1 Hypoxia-ischemia as a model of large hemispheric stroke

2.2 Aims and hypotheses

2.2.1 General aims

2.2.2 Specific hypotheses

Chapter 3

3 Methods

3.1 Animals

3.2 Hypoxia-Ischemia (HI)

3.3 Behavioral seizure monitoring and treatment

3.4 Intracranial electroencephalographic (EEG) recordings

3.4.1 Surgery and electrode implantation

3.4.2 Quantification of EEG activity

3.5 Telemetry recording

3.6 Doppler measurements of cerebral blood flow

3.7 Histology

3.8 In vitro electrophysiology

3.9 Statistical analyses

Chapter 4

4 Results I: HI induces mortality and structural brain injury

4.1 HI episodes and resulting mortality

4.2 Histological assessments of brain injury at later post-HI times

4.3 TUNEL and FluroJade staining reveal subtle brain injury

4.4 Summary of chapter 4
9 Results VI: Can EEG activity predict seizures? ................................................................. 124

9.1 Profound EEG suppression in animals with seizures ..................................................... 124
9.2 Longer duration of EEG suppression in animals with seizures ........................................ 125
9.3 Analysis of power spectral alterations in animals with seizures ..................................... 130
9.4 EEG suppression in animals without post-HI seizures .................................................. 135
9.5 Summary of chapter 9 ...................................................................................................... 138

Chapter 10 .......................................................................................................................... 139

10 Discussion ......................................................................................................................... 139

10.1 Modes of post-HI brain injury ....................................................................................... 139
10.2 Possible mechanisms of HI-induced brain injury ......................................................... 140
10.2.1 How does combining unilateral occlusion of the right common carotid artery with respiratory hypoxia result in brain injury? .......................................................... 142
10.2.2 How does HI result in panhemispheric brain injury? ................................................. 145
10.2.3 Why are some animals resistant to panhemispheric infarction following HI? .... 146
10.3 EEG activity as a marker for post-HI outcome .............................................................. 146
10.4 Main features and pathological significance of post HI seizures .............................. 150
10.5 Mortality, brain injury, and seizures .......................................................................... 150
10.6 EEG activity during post-HI seizures ........................................................................... 153
10.6.1 Non-convulsive EEG discharges .............................................................................. 153
10.6.2 Are post-HI convulsive seizures subcortical in origin? ........................................... 154

Chapter 11 .......................................................................................................................... 159

11 Conclusions, novelty, and outlook .................................................................................. 159

11.1 Main conclusions ......................................................................................................... 159
11.2 Novelty ........................................................................................................................ 160
11.3 Future experiments ....................................................................................................... 161
11.3.1 Resolving the mechanisms of post-HI brain injury ............................................... 161
11.3.2 Explaining the variability in post-HI outcome.............................................163

11.3.3 Determining the source of post-HI motor seizures ........................................164

References..........................................................................................................................166

Appendix I. Cerebral collateral flow: a review of the clinical and experimental impact ....179

Appendix II. Unilateral occlusion of the right common carotid artery does not affect EEG parameters in C57BL/6 mice .................................................................198
List of Figures

Figure 1. Non-convulsive seizures following middle cerebral artery occlusion in rats............... 17

Figure 2. Hypoxia-induced seizures in juvenile mice.......................................................... 35

Figure 3. Histological outcomes following hypoxia-ischemia............................................. 47

Figure 4. Quantification of post-HI brain injury.................................................................... 49

Figure 5. TUNEL and FluoroJade assessments of post-HI injury......................................... 53

Figure 6. Quantification of TUNEL and FluoroJade signals in post-HI mice..................... 55

Figure 7. HI-induced changes in cerebral blood flow............................................................ 60

Figure 8. HI-induced changes in body temperature.............................................................. 63

Figure 9. Rectal Temperature during HI in anesthetized mice............................................. 65

Figure 10. HI-induced changes in heart rate........................................................................ 69

Figure 11. Brain region-specific and behavioral state-dependent EEG signals in adult mice.... 74

Figure 12. EEG signals in a mouse with clear ipsilateral brain injury post-HI.................... 77

Figure 13. EEG signals in a mouse with minimal ipsilateral brain injury post-HI.............. 79

Figure 14. EEG signals in a sham-control mouse................................................................. 81

Figure 15. Ipsilateral EEG amplitude as a measure of HI-induced EEG suppression......... 84

Figure 16. Ipsilateral EEG variance as a measure of HI-induced EEG suppression.......... 86

Figure 17. Contralateral EEG amplitude is minimally altered by HI.................................. 89

Figure 18. Contralateral EEG variance is minimally altered by HI...................................... 91

Figure 19. Ipsilateral EEG RMS as a measure of HI-induced EEG suppression.............. 94
Figure 20. Disruption of hippocampal EEG theta rhythms in post-HI mice.................................97
Figure 21. Quantification of hippocampal theta rhythms in post-HI mice.................................99
Figure 22. In vitro electrophysiological assessments of HI brain injury.....................................102
Figure 23. Early-onset seizures are closely associated with infarction and mortality...............108
Figure 24. Treatment with anticonvulsants reduces seizure incidence and mortality...........112
Figure 25. Non-convulsive ipsilateral EEG discharges during and following HI..................116
Figure 26. EEG activities associated with post-HI motor seizures with wired EEG recordings119
Figure 27. Wireless telemetry recording during post-HI seizures reveals no EEG discharges ..121
Figure 28. EEG suppression in mice with or without post-HI motor seizures.......................126
Figure 29. Quantification of EEG suppression in mice with or without post-HI seizures........128
Figure 30. Dominant EEG frequencies in animals with or without post-HI seizures.............131
Figure 31. EEG dominant power in animals with or without post-HI seizures.....................133
Figure 32. EEG suppression in animals lacking post-HI seizures.............................................136
List of Appendices

Appendix I. Cerebral collateral flow: a review of the clinical and experimental impact ..........179

Appendix II. Unilateral occlusion of the right common carotid artery does not affect EEG parameters in C57BL/6 mice .................................................................198
Chapter 1

“The history of science, like the history of all human ideas, is a history of irresponsible dreams, of obstinacy, and of error. But science is one of the very few human activities – perhaps the only one – in which errors are systematically criticized and fairly often, in time, corrected. This is why we can say that, in science, we often learn from our mistakes, and why we can speak clearly and sensibly about making progress there.”

— Sir Karl Popper

1 Introduction

This thesis revolves around a simple theme; namely, seizures are enigmatic consequences of stroke. Still, a biological curiosity cannot form the sole basis of a biomedical research project. There is, of course, clinical relevance in examining the relationship between seizures and stroke. Hence, in the following pages I will first begin with a separate discussion of strokes and seizures, treating them as independent ‘disorders’. I will then follow this up with an exposition of the complex and poorly understood relationship that binds these two ‘disorders’ together.

1.1 A primer of stroke

References to what we today refer to as stroke date back almost 2400 years in the writings of Hippocrates, though in his time stroke was referred to as apoplexy, which, roughly translated from Greek, means ‘struck down by violence’ (Paciaroni and Bogousslavsky 2009). Today, stroke affects approximately 20 million people worldwide, and it is the second leading cause of death and the primary cause of disability in adults (Truelsen and Bonita 2009). In its modern usage, the word ‘stroke’ is a generic term that unifies a number of heterogeneous underlying disease states that ultimately induce acute cerebrovascular dysfunction, brain injury, disability, and a medley of medical complications that can result in death.

It is standard in both clinical and experimental practice to broadly divide strokes into two types: namely, ‘ischemic’ and ‘hemorrhagic’. Approximately 80 to 85% of strokes are classified as
‘ischemic’; these result from a focal interruption of blood flow in one or more of the major brain arteries or their superficial or perforating branches (De Freitas et al. 2009; Kumaral et al. 2009). The usual culprit in this focal interruption is the formation of a local (thrombotic occlusion) or circulating (thrombo-embolic) blood clot (Hand and Davis 2009). A number of underlying disease conditions can ultimately result in ischemic stroke, including atherosclerosis, atrial fibrillation, and damaged blood vessels (Hand and Davis 2009; Kumaral et al. 2009); however, despite adequate investigations, approximately 20% of ischemic strokes are of unknown etiology (Hand and Davis 2009).

The remaining 15 to 20% of strokes are classified as ‘primary hemorrhagic’, and most commonly arise following either trauma or the rupture of an aneurysmal dilation on a blood vessel, ultimately resulting in bleeding into the brain (Kelly et al. 2009; Viswanathan and Greenburg 2009). Other causes of hemorrhage include vascular malformations, bleeding tumors, coagulation defects, and amyloid angiopathy (Kelly et al. 2009; Viswanathan and Greenburg 2009). Moreover, ischemic strokes can also result in secondary hemorrhage due to hemorrhagic transformation of infarcted brain tissue (Viswanathan and Greenburg 2009). Finally, relatively rare forms of stroke are venous in origin due to blood clots in the dural sinus or cerebral veins (Canhao et al. 2009).

Thus, not all strokes are the same, for there are multiple underlying diseases that can contribute to stroke, and there are various manifestations of stroke in individuals.

The reduction of arterial cerebral blood flow during ischemic stroke deprives brain cells of oxygen and glucose, consequently halting cellular metabolism, and this leads to the death of brain cells through a complex interplay of ionic imbalances, edema, inflammation, oxidative stress, and the buildup of neurotoxic concentrations of excitatory amino acids such as L-glutamate (Lipton 1999; Somjen 2001; Krnjević 2008). In hemorrhagic stroke, there are the added complications of the exposure to toxic blood products, mass effects, and vasospasm (Viswanathan and Greenburg 2009).

Glutamate, in particular, is released excessively from neurons and glia during cerebral ischemia, and it has received much attention as a causal agent in brain cell death: it is the main player in what is referred to as ‘excitotoxicity’ or the ‘excitotoxic hypothesis’- terms originally coined by John Olney in 1978 (Olney and de Gubareff 1978). This hypothesis holds that elevated levels of
extracellular glutamate result in an over-stimulation of glutamate receptors, particularly the ionotropic N-methyl-D-aspartate (NMDA) receptor, resulting in cellular influx of ionic calcium, leading to calcium overload, which triggers multiple downstream pathways that ultimately result in cell death through irreversible damage to cellular structures and intracellular organelles (Sattler and Tymianski 2001). However, virtually every glutamate receptor has been implicated in excitotoxicity, and this pathological process has been implicated not only in cerebral ischemia but also in trauma, epilepsy, and a number of other neurodegenerative diseases (Sattler and Tymianski 2001).

The above factors lead to brain injury following stroke, and this consequently can result in neurological impairment, disability, and a reduced quality of life (Adams and Layden 2009). In addition, it is important to note that the acute phase of stroke is a clinical emergency that can lead to mortality or impede recovery through a number of medical complications, including increased intracranial pressure, brain herniation, cardiac dysfunction, pulmonary complications, and cerebral seizures (Adams et al. 2007; Selim 2009).

Currently, the only approved pharmacological therapy for acute ischemic stroke is the intra-arterial or intra-venous administration of a thrombolytic agent within four hours of stroke onset (Adams et al. 2007; Schellinger et al. 2009; del Zoppo 2010). Such a strategy lyse blood clots and re-establishes cerebral blood flow by vascular recanalization. Thrombolytic therapy was initially developed for the treatment of myocardial infarction and was later used for acute ischemic stroke. In particular, human recombinant tissue plasminogen activator (rt-PA) is currently used for treating acute ischemic stroke, though other drugs are being developed (Stroke Study Group 1995; Adams et al. 2007; Schellinger et al. 2009). It should be noted, however, that acutely removing blood clots does not necessarily alleviate the underlying disease that led to clot formation in the first place. Moreover, the limited therapeutic time-window of this approach is a major concern and poses challenges in clinical practice. In addition, thrombolytic and anticoagulant therapies are known to be associated with an increased risk of hemorrhagic transformation, particularly if administered at late stages following a stroke (del Zoppo 2010). Nonetheless, the development of rt-PA therapy in conjunction with advances in stroke diagnosis and improvements in the infrastructure and efficiency of acute stroke care have undeniably improved post-stroke outcome (Dirnagl 2006).
In addition to vascular recanalization, decades of intense basic and clinical stroke research have fueled the development of numerous putative interventions aimed at providing neuroprotection in the acute phase of stroke; that is to say, strategies that directly reduce, reverse, or restrict brain cell death following a stroke. Supplementing rt-PA therapy with a viable neuroprotective strategy is an attractive prospect that would be of great clinical value in minimizing brain injury following ischemic stroke.

The process of developing drugs or interventions in the laboratory followed by their implementation in clinical practice is usually referred to as ‘translational’ or ‘bench-to-bedside’ research. Experimental research in animals has yielded over a thousand potential pharmacological agents or interventional approaches that have shown great promise in protecting brain cells in experimental stroke studies, and many of these have been translated to clinical trials (Adams et al. 2007; del Zoppo 2010). Perhaps the most well-known targets are glutamate receptors, which, as indicated above, are strongly implicated in playing a role in excitotoxicity; moreover, other non-pharmacological interventions such as hypothermia have been investigated (Adams et al. 2007; Macleod et al. 2010). Yet, to date, there is currently no neuroprotective intervention for acute stroke: all clinical trials have notoriously failed to deliver (Adams et al. 2007; del Zoppo 2010). This failure has understandably sparked intense debate, and a number of potential reasons for what has been derisively dubbed as ‘loss in translation’ have been put forth (STAIR 1999; Dirnagl 2006; Sena et al. 2007; Crossley et al. 2008; Macleod 2009; del Zoppo 2010; Minnerup et al. 2010). One of the central tenets of this thesis is that seizures are intimately associated with stroke, and that taking seizures into consideration may aid in the development of, or at the very least may supplement, neuroprotective and thrombolytic treatments for stroke.

1.2 Elementary principles of seizures

In the previous section I mentioned that stroke can result in a medical complication referred to as seizures. And this association is the main focus of this thesis. However, before I embark on an exposition of this complex association, I ought to describe some of the pertinent features of seizures and attempt to clarify the vast, and, at times, confusing terminology associated with these intriguing phenomena.
1.2.1 Towards a mechanistic definition of seizures and epilepsy

Descriptions of the symptoms of cerebral seizures date back to antiquity\(^1\). However, up until the late 19\(^{th}\) century, virtually nothing was known about these events, and they were frequently associated with supernatural causes, such as divine intervention or demonic possession (Temkin 1971; Magiorkinis et al. 2010); indeed, the term ‘seizure’ connotes unwilled possession. The basis, however, for the modern understanding of seizures and epilepsy is rooted, in the main, in an intuitive conjecture by John Hughlings Jackson, the father of British neurology, who in 1870 wrote (Jackson 1870; Somjen 2004; Iniesta 2011):

“A convolution is but a symptom, and implies that there is an occasional, an excessive, and a disorderly discharge of nerve tissue on muscle. This discharge occurs in all degrees; it occurs with all sorts of conditions of ill health, at all ages, and under innumerable circumstances.”

Decades of research have since confirmed Jackson’s conjecture; yet, to this day, precisely defining a seizure has proven to be a difficult task. A slightly more modern definition is as follows (Somjen 2004):

“Epileptic (or epileptiform) seizures involve the episodic, uncontrolled, excessive synchronous discharge of groups of central neurons, and epilepsy is a chronic condition characterized by repeated, unprovoked epileptic seizures.”

Further, the International League Against Epilepsy (ILAE) has agreed on the following definitions (Fisher et al. 2005):

“An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.”

\(^1\) The earliest known written record describing seizure symptoms is from Mesopotamia in 2000 B.C, which, roughly translated, states: “his neck turns left, his hands and feet are tense and his eyes wide open, and from his mouth froth is flowing without his having consciousness”. The condition was promptly diagnosed by the author of this text as “the hand of sin” imposed upon the unfortunate victim by the God of the Moon (Magiorkinis et al. 2010). As we shall see later, this is probably a description of generalized tonic-clonic convulsions, and is but one a multitude of seizure types.

In the times of ancient Greece, it is believed that Hippocrates was the first to argue for a non-supernatural cause of seizures, which was commonly referred to in his time as the ‘falling sickness’ (Temkin 1971; Magiorkinis et al. 2010).
“Epilepsy is a chronic disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiological, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure.”

It is useful to further divide epilepsy into two broad categories: symptomatic (or secondary) and essential (or idiopathic) (Engel 2001; Somjen 2004; Fisher et al. 2005; Noachtar and Peters 2009). Symptomatic epilepsy is the result of an identifiable brain lesion or disease that, for as yet poorly understood reasons, predisposes the brain to epileptic seizures. Idiopathic epilepsy, on the other hand, refers to an unclear origin or cause, though genetic defects in the structure or function of various ion channels may play a role.

So much for definitions of seizures and epilepsy.

On a final note, it is important to understand that seizures are not exclusively associated with epilepsy. Seizures may be transiently and acutely brought about in non-epileptic patients by a number of insults such as infections, fever, trauma, stroke, drug intoxication, drug withdrawal, hypoglycemia, and sleeplessness; in fact, more than 5% of all people are said to suffer from at least one epileptic seizure during their lives (Somjen 2004).

1.2.2 How do seizures arise?

To my knowledge, a unified explanatory theory that explains how seizures arise does not presently exist. Thus any attempt to answer this question will be incomplete at best. However, a number of more or less universally accepted concepts have emerged from decades of research, and these have provided a useful conceptual framework within which to explain and investigate seizures. These concepts are: 1) the hijacking of intrinsic oscillatory systems 2) hyperexcitability 3) hypersynchrony 4) spread and recruitment 5) termination.

I shall now very briefly address these issues as they will be useful when discussing the unknown mechanisms for the genesis of post-stroke seizures.

1) The hijacking of intrinsic oscillatory systems

The above definitions (see 1.2.1) described seizures as involving neuronal activity that is episodic and synchronous. It should be noted, however, that synchronous neuronal activity is not
a peculiarity singular to epileptic seizures; rather, it appears to be a fundamental characteristic of neuronal networks in the ‘normal’ brain. Coordinated spontaneous firing of large numbers of central neurons occurs under normal conditions. Notable examples can be found in the thalamocortical network and within the hippocampal CA3 sector (Buzsáki 2006). For instance, slow-wave sleep results in the emergence of synchronous activity in the cerebral cortex and hippocampus that arguably fits into the above definition of seizures; even simply closing the eyes results in the emergence of synchronized (alpha) oscillations. It has therefore been argued that the default state of brain activity is, in fact, rhythmic and oscillatory (Buzsáki 2006); and that a seizure may represent a “hijacking” of this default state: “neurons that fire together also conspire together” (Beenhakker and Huguenard 2009). The point here, however, is that seizures do not arise from nothing – *ex nihilo nihil fit* – but are likely an aberrant consequence of the intrinsic oscillatory circuits in the brain. The key term here being aberrant, such that it interferes with normal brain function or, as discussed below, leads to brain damage and possibly death.

2) **Hyperexcitability**

The cellular mechanisms by which seizures are generated, spread, and terminated are still a matter of debate. Regarding seizure genesis, a number of useful explanatory concepts have emerged. One such concept is neuronal hyperexcitability, and this is, by and large, universally accepted as being a prerequisite for seizure genesis (Westbrook 2000; Somjen 2004; Beenhakker and Huguenard 2009; Dichter 2009). In general, excessive excitation, decreased inhibition, or a combination of both are hypothesized to precipitate seizures.

A vast number of cellular alterations can lead to increased neuronal excitation, and these can range from simple disturbances to the ionic milieu to more complex alterations in ion channel function and structural changes in synapses and neuronal connections (Somjen 2004). Importantly, the hyperexcitability viewpoint has formed the basis of decades of experimental research; and, incidentally, underlies the proposed modes of action of a wide spectrum of anticonvulsant drugs, which are believed to either reduce neuronal excitability or boost inhibition.
3) Hypersynchrony

Yet hyperexcitability alone cannot explain how a seizure arises: the definitions above employed the term ‘synchronous’. Thus, a second key concept in seizure genesis is synchronicity, which is generally defined as coordinated neuronal activity (Westbrook 2000; Somjen 2004; Beenhakker and Huguenard 2009; Dichter 2009). Generally speaking, this means that many neurons or synapses need to be excited simultaneously for a seizure to occur. However, the mechanisms of synchronized synaptic activity during seizures are poorly understood. A number of factors have been postulated, including a break-down of feed-forward or feed-back inhibition, synchronization through neuronal gap junctions, and recruitment via interconnected circuitry. Intuitively, it would be expected that synchronized synaptic activity is a prerequisite for seizures; however, seizures can also occur in the absence of synaptic transmission. For example, low concentrations of extracellular calcium ions, at concentrations low enough to inhibit synaptic transmission, can result in seizure-like activity in isolated brain slices (Jefferys and Haas 1982; Taylor and Dudek 1982; Perez-Velazquez et al. 1994). Seizures, then, likely involve complex synaptic and non-synaptic interactions between principal cells, interneurons, and, possibly, astrocytes.

4) Spread and Recruitment

An important characteristic of seizures is that they can sometimes start in a localized region, commonly referred to as a ‘focus’, and then invade and recruit surrounding or distant brain regions; however, the mechanisms by which seizures spread remain elusive (Westbrook 2000; Somjen 2004; Beenhakker and Huguenard 2009; Dichter 2009). Several factors have been implicated, including a spread of excitation via synaptic pathways, ephaptic conduction, diffusion of neurotransmitters or ions, and spread through the astrocytic pseudo-syncytium (see above references).

5) Termination

Finally, there is the issue of seizure termination: if seizures are self-generating, rhythmic cellular events, then why are they ephemeral and self-terminating? A number of factors have been implicated in seizure termination, including inactivation of ion channels, depletion of releasable
neurotransmitter pools, release of endogenous inhibitory modulators, and cerebral acidosis (which is inhibitory) (Somjen 2004). Yet it is unclear as to how seizures actually terminate.

In summary

To sum up, a conceptual framework for understanding seizure genesis, spread, and termination is currently in place. This framework has been made possible by numerous detailed neurophysiological investigations in humans and in animal models as well as theoretical studies using computational models, which have collectively examined the underlying cellular and synaptic events that occur during a seizure. These issues are here greatly simplified for the sake of brevity. Nonetheless, there are still many unanswered questions regarding these peculiar brain events – in fact, there is still no unified answer to the pertinent question: how do seizures arise?

1.2.3 The electroencephalogram and seizures

The synchronized activity of neurons during a seizure can be detected by the electroencephalogram (EEG), a device that measures electrical activity in the brain\(^2\). The EEG is believed to reflect the synchronized electrical activity of a large number of neurons and synapses. These signals are recorded extracellularly with electrodes placed either on the scalp, epidurally, or intracranially (Somjen 2004; Buzsáki 2006). The generation of these signals requires not only the synchronized activity of a large number of neurons and synapses but also a certain spatial geometry of cells and synapses that permits the development of an appreciable electric field. Under normal conditions, the probable major contributors to the EEG signal are excitatory and inhibitory synaptic potentials, with a relatively lesser contribution from neuronal action potentials and dendritic or glial slow potentials (Somjen 2004; Buzsáki 2006).

The EEG is considered to be the primary method for detecting and confirming seizures (Markand 2003; Jordan 2004; Somjen 2004; Friedman et al. 2009a). Seizures result in ‘electrical storms’ in the EEG – conspicuous, synchronized, large amplitude events imposed upon background EEG

\(^2\) Hans Berger is usually credited with developing this technique in the late 1920s (Berger 1929; Buzsáki 2006). Using a string galvanometer, Berger was able to measure voltage changes from the scalp, and he is usually credited with discovering both the human alpha rhythm alluded to above and the characteristic spike-wave discharges associated with absence seizures described below. On a side note, the main motive behind Berger’s research was not neurophysiological: it was occult. He believed that the electromagnetic fields created by the brain could be carriers for telepathy.
activity that would be obvious even to the untrained eye. Such ‘electrical storms’ are commonly referred to as ‘ictal events’ or ‘ictal discharges’, and they may or may not be associated with an obvious behavioral correlate. In addition to ictal activity, a diverse group of EEG abnormalities, collectively referred to as ‘interictal’ activity, may be observed in EEG recordings, and may herald the onset of an impending ictal event. Interictal activity most commonly manifests in the form of isolated EEG spikes or short bursts of aberrant EEG activity. Finally, suppression in background EEG activity may be observed following a seizure, and this is referred to as ‘post-ictal depression’. Importantly, seizures are relatively rare events, even in epileptic patients; hence, in order to adequately capture electrographic seizures, long-term continuous EEG monitoring is required, and this results in obvious logistical problems.

1.2.4 A note on seizure classification

There are many different types of seizures, and it is important to make a clinical distinction as different seizures may arise through different cellular mechanisms, involve different brain regions, and may require different treatment strategies. Seizures can be broadly classified according to behavioral manifestations, brain regions involved, and EEG activity (Westbrook 2000; Engel 2001; Burnham et al. 2002; Somjen 2004; Fisher et al. 2005; Noachtar and Peters 2009). The terminology is vast, perplexing, continuously evolving, and a comprehensive, consolidated, and universally agreed upon vocabulary does not presently exist. For the purposes of this thesis, I would like to distinguish seizures in terms of overt behavioral manifestations and the size of the brain region involved. These distinctions, though clinically valid, are here simplified as a prelude to the forthcoming discussion of the complex field of post-stroke seizures.

Thus, seizures are ‘convulsive’ if overt, involuntary muscular spasms are observed and ‘non-convulsive’ in their absence; that is to say, in the latter case, that seizures may be subtle, subclinical, and conclusively detected only through the EEG. Furthermore, seizures are referred to as ‘partial’ if a limited brain area is involved or ‘generalized’ if a major proportion of both hemispheres participate. Partial seizures may or may not be associated with convulsions

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3 Examples of ictal discharges in EEG recordings in rodents can be found in Figure 1 (p. 17), Figure 2 (p. 35), and Figure 25 (p. 117).
depending on the precise brain region involved. Partial seizures can further be divided into simple (if consciousness is retained) and complex (if consciousness is impaired). Importantly, partial seizures usually begin in a localized ‘seizure focus’ and may recruit larger brain areas, and therefore evolve into a ‘secondary generalized’ seizure, which contrasts it to a ‘primary generalized’ seizure. Primary generalized seizures are particularly baffling as they appear to start diffusely in bilateral brain areas at the same time.

Primary and secondary generalized seizures are of interest in this thesis. Generalized seizures have classically been defined as involving large portions of both cerebral hemispheres, and they can be broadly divided into non-convulsive and convulsive types. There are several types of generalized seizures; however, two antipodal examples are sufficient for this thesis. An example of a non-convulsive generalized seizure is the typical ‘absence’ or ‘petit mal (little evil)’ seizure (Westbrook 2000; Engel 2001; Cruneli and Lerescue 2002; Somjen 2004; Noachtar and Peters 2009). These seizures can be mild, in the sense that they cause a momentary lapse of consciousness with occasional twitching but without falling or major convulsions. These types of seizures are usually observed in young epileptic children, and are associated with non-convulsive ictal EEG activity that is characterized by bilateral, synchronous, rhythmic 2.5 to 4Hz spike-wave discharges. The generation of these spike-wave discharges is believed to arise through a concerted interaction between the cerebral cortex and thalamus, which normally acts to drive rhythmic neuronal activities during slow-wave sleep.

On the opposite end of the spectrum, generalized seizures can also result in ‘generalized tonic-clonic convulsions’, also referred to as ‘grand mal (big evil)’ seizures (Westbrook 2000; Engel 2001; Somjen 2004; Noachtar and Peters 2009). Grand mal seizures are dramatic events, and they are associated with a loss of consciousness, falling, followed by tonic phases in which there are simultaneous muscular spasms and by clonic phases in which the body jerks rhythmically; this is then usually followed by lethargy, muscular soreness and, in some cases, coma (Theodore et al. 1994; Jobst et al. 2001; Somjen 2004). Generalized tonic-clonic seizures can involve the cerebral cortex and subcortical structures, yet both the mechanisms by which these seizures arise and the brain regions recruited are more heterogeneous and poorly understood in comparison to the absence seizures described above. Tonic phases of generalized seizures are characterized by a barrage of ictal EEG discharges, while clonic phases are characterized by synchronous neuronal activity in sync with the clonic movements of the body.
There are a number of variations to the above described events, as well as a number of intermediaries. However, the main point here is that, despite involving large areas of the brain, individual generalized seizures can exhibit strikingly different EEG activities and can arise through different cellular mechanisms.

Generalized tonic-clonic seizures are the most dangerous of all seizures, and may result in mortality and injury through accidents and falls, vertebral fractures, muscular injury, cardiac and respiratory dysfunction, and brain damage (Nei and Bagla 2007; Jehi and Najm 2008). A clinically important aspect of seizures is ‘status epilepticus’, which has been generally defined as continuous or intermittent seizures lasting 30 minutes or longer (Meierkord et al. 2010). Generalized tonic-clonic convulsive status epilepticus is a life threatening emergency, and requires immediate anticonvulsant therapy (Meierkord et al. 2010).

1.2.5 Seizures summarized

The above brief introduction to seizures serves to highlight the main aspects relevant to this thesis. In summary, seizures involve the episodic, uncontrolled, excessive, synchronous discharges of groups of central neurons. Importantly, seizures can be convulsive or non-convulsive. The mechanisms by which seizures arise, spread, and terminate are enigmatic, multifaceted, and complex. However, the concepts of hyperexcitability and hypersynchrony are useful starting points in investigating the cellular mechanisms of seizure genesis. Moreover, strictly speaking, there is no such thing as a ‘typical seizure’: the anatomical origin, size of the brain regions involved, behavioral manifestations, electrographic characteristics, pharmacology, and cellular mechanisms are highly variable. Finally, seizures may be transiently precipitated in non-epileptic patients during the acute phases of a variety of brain insults; such insults are a double-edged sword as they may, in turn, result in the development of secondary epilepsy in later life. One such brain insult is stroke, and it is this topic that will dominate the remainder of this thesis.

1.3 General introduction to post-stroke seizures

John Hughlings Jackson first described seizures as a consequence of stroke in the 1800s (Taylor et al. 2003). Today, stroke is the most commonly identified ‘cause’ of seizures in the adult and aging populations (Camilo and Goldstein 2004; Feleppa et al. 2006; Bladin and Bornstein 2009;
Menon and Shorvon 2009). It is well established that both ischemic and hemorrhagic stroke can result in seizures in humans. The reported incidences of seizures following stroke vary dramatically, and can range from 2 to 48% of stroke patients, with the majority of these seizures occurring within 24 hours of stroke; furthermore, status epilepticus has been reported to occur in 0.14% to 13% of acute stroke cases (Camilo and Goldstein 2004; Feleppa et al. 2006; Bladin and Bornstein 2009; Menon and Shorvon 2009).

Post-stroke seizures are generally classified according to the time of onset following stroke. An arbitrary cut-off time of 2 weeks is generally agreed upon to delineate ‘early-onset’ seizures, while seizures occurring months or even years after stroke are designated as ‘late-onset’; and a similar temporal pattern has been observed in animal models of stroke (Kelly 2007; Pitkänen et al. 2007; Epsztein et al. 2008; Bladin and Bornstein 2009). The main reason for this temporal division is that the early and late-onset seizures are believed to be distinct events, with a different underlying pathogenesis (Epsztein et al. 2008).

Late-onset seizures are believed to result from acquired epilepsy secondary to brain injury (Kelly 2007; Epsztein et al. 2008). This is believed to be the consequence of complex structural and synaptic alterations that arise secondary to brain lesions, and these alterations have been suggested to lead to heightened excitability and neuronal synchrony. Examples of such reported structural changes include gliosis, alterations in neuronal membrane properties, and the sprouting of new excitatory synaptic connections. However, the mechanisms by which these structural alterations arise and lead to seizures remain to be clearly elucidated. On the other hand, early-onset seizures are considered to be transient events associated with the early phases of stroke. It has been suggested that early-onset seizures are caused by acute, transient metabolic and ionic derangements in the brain, leading to “electrically hyperexcitable” tissue and an elevated probability for seizures (Hartings 2003; Kelly 2007; Bladin and Bornstein 2009).

The early-onset seizures are the main focus of this thesis, and more details regarding possible mechanisms will be described below (see 1.6). Although late-onset seizures are clinically relevant events that warrant further investigation, I will not discuss them further. Interested readers can consult a number of excellent reviews on the subject (Kelly 2007; Epsztein et al. 2008; Bladin and Bornstein 2009).
1.4 Early-onset post-stroke seizures in humans

The majority of post-stroke seizures occur within 24 hours of stroke and are generally considered to be a clinical emergency (Camilo and Goldstein 2004; Jordan 2004; Feleppa et al. 2006; Bladin and Bornstein 2009; Menon and Shorvon 2009). Regarding behavioral manifestations, early onset-seizures can range from being sub-clinical to outright convulsive. Regarding classifications, approximately 50-90% of seizures are described as simple partial motor seizures that occasionally secondarily generalize (Bladin and Bornstein 2009; Menon and Shorvon 2009). However, the accuracy of such traditional classifications may be called into question, as the diagnosis of a seizure may be complicated by a number of stroke-related factors such as motor dysfunction, disorientation, and unconsciousness, which is further confounded if EEG recordings are not implemented.

Yet the role of the EEG in assessing post-stroke seizures remains controversial. Some studies have found continuous EEG monitoring in the hours and days following a stroke to be useful in diagnosing the occurrence of non-convulsive status epilepticus (Jordan 2004). However, the descriptions of ictal EEG alterations following acute stroke can be non-specific and are usually focal, and aberrant EEG activity is sometimes associated with a number of interictal EEG abnormalities, such as periodic lateralizing epileptiform discharges (PLEDs), frontal intermittent rhythmic delta activity (FIRDA), and sharp wave activity (Jordan 2004; Bladin and Bornstein 2009; Menon and Shorvon 2009). The utility of the EEG in predicting or diagnosing seizures related to stroke remains to be determined; in particular, some authors do not consider the EEG as being useful in assessing post-stroke seizures nor in providing a sole basis for anticonvulsant therapy (Bladin and Bornstein 2009). Notably, continuous EEG monitoring is a challenging logistical problem, particularly for critically ill patients in the intensive care unit (Friedman et al. 2009a).

Despite the common observation of early-onset seizures following stroke, the impact of seizures on post-stroke outcome remains contentious (Camilo and Goldstein 2004; Jordan 2004; Feleppa et al. 2006; Bladin and Bornstein 2009; Menon and Shorvon 2009). For example, two studies have found no association between early-onset seizures and mortality (Reith et al. 1997; Labovitz et al. 2001), while others have identified early-onset seizures as a risk factor for mortality and poor neurological outcome (Bladin et al. 2000; Burneo et al. 2010). However,
mortality should not be the ultimate measure of efficacy in the management of seizure disorders. Seizure freedom is the goal and the most important indicator of quality of life for the patient. Furthermore, it is difficult to evaluate the impact of early seizures on the degree of neurological recovery following stroke. Despite the unclear effects on outcome, the current medical recommendation is to treat post-stroke seizures with anticonvulsants, especially if status epilepticus is observed; however, prophylactic treatment is not recommended nor carried out in clinical practice (Adams et al. 2007; Bladin and Bornstein 2009). Although post-stroke seizures respond well to anticonvulsant medication, the influence of such treatments on improving post-stroke neurological outcome are unclear and a standardized treatment regimen does not currently exist (Kwan and Wood 2010). Moreover, some anticonvulsant drugs can result in adverse reactions, particularly in the elderly (Perucca et al. 2006) and this may further complicate treatment and/or recovery.

Although, as described above, the cellular mechanisms for the generation of early post-stroke seizures are currently unclear, a number of risk factors have been implicated (Bladin and Bornstein 2009; Menon and Shorvon 2009). A cortical site of injury, larger area of brain injury, a cardiac embolism, hypertension, raised serum cholesterol, and left ventricular hypertrophy have all been implicated as potential risk factors for the development of early post-stroke seizures. However, the precise contribution of these factors to seizure genesis is currently unknown.

In summary, the incidence of early post-stroke seizures is imprecisely estimated. The reported behavioral manifestations of these seizures are highly variable. The impact of post-stroke seizures on neurological outcome and mortality following stroke is not well-understood. A clear documentation of the EEG alterations associated with post-stroke seizures is currently unavailable. An understanding of the anatomical origin of post-stroke seizures is lacking. The contributions of identified risk factors to the development of post-stroke seizures and the mechanisms of post-stroke seizure genesis are currently unknown.

It is difficult to interpret the results from human epidemiological studies for a number of reasons. Differences in seizure identification criteria, variable follow up times, the sporadic use of EEG recordings, community-based versus hospital-based studies, different sample sizes, heterogeneity of stroke sub-types, and a number of potential co-morbidities, can all contribute to variable and unclear results. Most of these difficulties have been acknowledged by others (Camilo and
It is clear, however, that strokes result in seizures, and that the majority of these seizures occur within 24 hours of stroke. Experimental studies in a controlled laboratory setting may shed light on the mechanisms and consequences of post-stroke seizures, and this topic will be addressed next.

### 1.5 Early-onset post-stroke seizures in rodents

Rodent models of ischemic and hemorrhagic stroke are believed to mimic the corresponding human conditions (Hossmann 2007; Durukan and Tatlisumak 2009), and they can lead to the development of both early and late-onset seizures when carried out in adult animals (Lee et al. 1997; Karhunen et al. 2005; Kelly 2007; Pitkänen et al. 2007; Epsztein et al. 2008). Late-onset seizures (i.e., epilepsy) following experimental cerebral ischemia have received much attention and have been reviewed elsewhere (Kelly 2007; Epsztein et al. 2008). Yet a comprehensive and in-depth review of the early-onset seizures following experimental focal cerebral ischemia is not available. Here I will review the current literature on early-onset seizures following experimental cerebral ischemia in rodents.

Middle cerebral artery occlusion (MCAO) is likely the most commonly employed experimental model of stroke in rats (Hossmann 2007; Durukan and Tatlisumak 2009). The procedure consists of a unilateral occlusion of the middle cerebral artery (MCAO) by a number of surgically invasive methods, and MCAO occlusion results in a primary region of damage to the ipsilateral parietal cortices and the basal ganglia. Though the precise extent and scope of damage varies according to the experimental parameters employed, MCAO can result in damage in up to two-thirds of the ipsilateral hemisphere (Hossmann 2007; Durukan and Tatlisumak 2009).

Several studies have reported that early-onset seizures occur within a few hours of MCAO in rats, with reported incidences ranging from 23-100% of challenged animals (Kudo et al. 1982; Reglodi et al. 2000; Lu et al. 2001; Wang et al. 2001; Shuaib et al. 2002; Hartings et al. 2003; Williams et al. 2004; Karhunen et al. 2005; Shabanzadeh et al. 2005; Williams et al. 2006). These seizures appear to be generalized in nature, and can be broadly divided into non-convulsive and convulsive generalized seizures. As described above (see 1.2.4), generalized seizures in humans are usually classified as convulsive or non-convulsive, and I shall adopt the same approach here.
Figure 1. Non-convulsive seizures following middle cerebral artery occlusion in rats
**Figure 1. Non-convulsive seizures following middle cerebral artery occlusion in rats**

Epidural EEG recordings from the ipsilateral parietal cortex following permanent middle cerebral artery occlusion (MCAO) in Sprague-Dawley rats. **A**, Raw EEG traces prior to and following MCAO. Note a strong suppression in EEG activity following MCAO. **B**, Raw EEG trace of two non-convulsive seizures (NCS, arrows) following MCAO. NCS events presented as repetitive spike and/or sharp wave discharges, and were not associated with motor convulsions i.e., these are non-convulsive seizures. **C**, Total EEG power prior to and following MCAO. Baseline fluctuations reflect shifts in EEG power associated with sleep-wake activity. Although total power was reduced following MCAO, increased EEG power was noted in association with NCS events (arrows). The majority of NCS events occurred within 1 to 3 hours of MCAO. Although not evident in this figure, post-MCAO non-convulsive seizures are generalized and can be detected bilaterally across large regions of the cerebral cortex (see main text for details). Figure modified from Williams et al (2006) with permission from the American Society for Pharmacology and Experimental Therapeutics.
1.5.1 Generalized non-convulsive early-onset post-ischemic seizures

By far, the most detailed assessment of post-MCAO seizures in rats was carried out by Hartings et al (2003). This study employed what is referred to as the intraluminal filament model of MCAO, which was first described by Koizumi et al (1986). The method employs the surgical advancement of a nylon suture through the internal carotid artery to the tip of the middle cerebral artery. Successful occlusion is usually confirmed via assessments of cerebral blood flow in the cortical regions supplied by this artery, though suppressed EEG activity has also been employed as a marker for successful ischemia. Ischemia induced in this fashion can be ‘transient’ if the suture is removed after 1-3 hours, or ‘permanent’ if it is left in place. The permanent strategy results in more extensive brain injury and higher incidences of mortality.

Utilizing the intraluminal method of MCAO, Hartings and colleagues (2003) observed post-MCAO seizures in up to 85% of challenged rats. The seizures occurred, on average, 30 minutes following MCAO and persisted up to approximately 3 hours later in animals with permanent MCAO. Interestingly, in animals exposed to a 2 hour transient MCAO, a similar incidence of seizures was also observed, but seizures did not extend into reperfusion. These seizures were associated with approximately one minute long bilateral spike-wave ictal discharges in epidural cortical EEG recordings, the majority of which had a focal onset in the region of the ipsilateral parietal cortex that then secondarily generalized to large regions of the cortex in both hemispheres (Figure 1). Additionally, other abnormal interictal EEG activities such as periodic lateralized epileptiform discharges and intermittent rhythmic delta activity were also noted. Importantly, the electrographic seizures were not associated with obvious motor manifestations or convulsions. According to the authors, “during seizures, animals exhibited restful, awake behavior with occasional ‘wet dog shakes’, or engaged in motor behaviors indistinguishable from those observed in the absence of ictal EEG patterns”. That is to say, these seizures are non-convulsive. My interpretation is that these seizures closely resemble generalized absence seizures (see 1.2.4) in both behavioral manifestations and EEG characteristics, though the authors did not explicitly make this allusion, choosing instead the broad term ‘non-convulsive seizures’.

Likewise, the observation of similar non-convulsive seizures following MCAO has been independently reported by others utilizing either the intraluminal filament (Lu et al. 2001) or
endothelin-1-induced spasm model of MCAO (Karhunen et al. 2005). The latter method involves the stereotaxic injection of the vasoconstrictor endothelin-1 into the middle cerebral artery through a craniotomy, and this results in binding of endothelin-1 to smooth muscle cells and subsequent arterial spasm and occlusion for up to 16 hours.

Moreover, subsequent follow up studies have demonstrated that a high incidence of non-convulsive seizures following MCAO in rats is associated with mortality and larger lesions. It has therefore been suggested that seizures may place a greater metabolic demand on ischemic brain tissue, thus leading to larger lesions and poorer post-ischemic outcome (Williams et al. 2004; Williams et al. 2006). However, in my opinion, this hypothesis remains to be fully tested. Moreover, others have failed to detect such a correlation (Lu et al. 2001). Nonetheless, a wide spectrum of anticonvulsants has demonstrated efficacy in reducing non-convulsive seizure incidence, brain injury, and mortality following MCAO in rats (Williams et al. 2004; Williams et al. 2006).

1.5.2 Generalized convulsive early-onset post-ischemic seizures

Interestingly, seizures have also been reported following thromboembolic models of MCAO (Kudo et al. 1982; Reglodi et al. 2000; Wang et al. 2001; Shuaib et al. 2002; Shabanzadeh et al. 2005). This model was originally described by Hill et al (1955), and typically involves the injection of either pre-formed autologous blood clots or artificial embolic materials into the middle cerebral artery. However, the extent of subsequent brain injury can be inherently variable compared to other MCAO models, and this model generally results in more extensive brain injury than MCAO with the intraluminal filament described above (DiNapoli et al. 2006; Hossmann 2007; Durukan and Tatlisumak 2009). Curiously, these studies have reported the occurrence of convulsive post-MCAO seizures; that is to say, seizures with clear motor manifestations. This observation is diametrically opposed to the above described studies utilizing the filament or endothelin-1 MCAO models (see 1.5.1 above). Unfortunately, a characterization of the convulsive seizures was not the main focus of these studies, and the reports of convulsions were non-specific and incidental. Moreover, as EEG recordings were not implemented during these convulsive seizures, a comparison to the above described detailed EEG assessments of non-convulsive post-MCAO seizures carried out by Hartings et al (2003) is not possible.
1.5.3 Early-onset seizures in other models of cerebral ischemia

The above discussion is primarily focused on the available literature on early-onset seizures following focal ischemic episodes in adult rodents. However, early-onset seizures have also been reported in animal models of global cerebral ischemia. Such ‘global ischemic’ models typically mimic clinical conditions such as coronary bypass surgery, cardiac arrest, and coronary artery occlusion (Hossman 2007; Epsztein et al. 2008). In particular, early-onset seizures have been reported following transient forebrain ischemia induced by the 4-vessel (bilateral carotid and vertebral arteries) occlusion method in rats (Schmidt-Kastner et al. 1989; Voll and Auer. 1991; Caruana et al. 2008) and following transient bilateral occlusion of the common carotid arteries in gerbils (Grazia Marciani et al. 1990). Furthermore, it has been demonstrated that rats subjected to transient global cerebral ischemia (induced by transient chest compression) exhibit a reduced threshold to audiogenic seizures (Iyer et al. 1995; Reid et al. 1996). However, the observation of seizures following transient global ischemia in rodents has explicitly not been reported by others (Armstrong et al. 1989; Buzsáki et al. 1999) and conceptually challenged (Heim et al. 1991; Winkler et al. 2001); and the incidence of seizures following cardiac arrest in humans is estimated to be approximately 1% (Goldstone et al. 2011), which is lower than that generally reported following stroke.

Additionally, it is well-established that cerebral ischemic/hypoxic episodes can cause seizures in neonatal/immature animals and humans (Jensen et al. 1992; Zupanc 2004; Comi et al. 2009; Wais et al. 2009; Bjorkman et al. 2010). However, the extent to which ischemic episodes in neonatal brains can be extrapolated to fully mature brains is unclear, as the developing brain has a different synaptic organization. Thus, as stroke is generally a disease of the elderly, such hypoxic/ischemic seizures in neonatal animals are not addressed here.

1.6 Possible mechanisms of early post-stroke seizures

As mentioned above, it has been suggested that early-onset seizures are caused by acute, transient metabolic and ionic derangements in the brain, leading to “electrically hyperexcitable” tissue and an elevated probability of seizures (Hartings et al. 2003; Kelly 2007; Bladin and Bornstein 2009). However, precisely what these derangements are, how they lead to hyperexcitability following cerebral ischemia, and exactly where and how this leads to seizures, have not been well-defined.
Brain regions most heavily supplied by an occluded artery are destined to form what is referred to as the necrotic ischemic core (Lipton 1999). The ischemic core is surrounded by a secondary region of hypoperfused tissue known as the penumbra. Collateral cerebral blood flow maintains a sufficient, though compromised, supply of arterial blood to the penumbra that allows this region to evade rapid injury, though it may succumb to delayed brain injury in the days following the ischemic episode. The penumbra is in turn surrounded by relatively normally perfused brain tissue. In the ischemic core, synaptic transmission is rapidly abolished and cellular damage is rapid and irreversible; however, a spill-over of potential ‘pro-hyperexcitable’ factors into the penumbra or surrounding regions may trigger a seizure.

For example, it has been demonstrated that anoxic/aglycemic episodes can result in a long-term potentiation (LTP) of glutamatergic synaptic transmission in the rodent hippocampal CA1 region in acute slice preparations in vitro (‘anoxic LTP’ hypothesis) (Hammond et al. 2004; Crepel et al. 2003; Krnjević 2008). LTP is considered as a cellular correlate of memory, and it has been extensively studied in the rodent hippocampus (Kandel 2000). In classical LTP in the hippocampal CA1 sector, high frequency tetanic stimulation of CA3 afferent axons results in repeated activation of postsynaptic AMPA receptors on CA1 pyramidal cell dendrites. The intense depolarization of the postsynaptic membrane relieves the magnesium blockade of NMDA receptors, resulting in the influx of calcium into dendritic spines. This then activates calcium-dependent kinases that are believed to increase the sensitivity and number of postsynaptic AMPA receptors that respond to glutamate, likely via direct phosphorylation of these receptors. The net consequence is enhanced glutamatergic transmission that lasts approximately 3–4 hours, and is referred to as ‘early LTP’. LTP can also extend for longer durations, and this likely involves de novo protein synthesis and structural changes in the synaptic machinery. As with classical tetanic LTP, the induction of anoxic LTP requires a persistent activation of NMDA receptors and a rise in intracellular calcium concentrations, as well as the activation of second messenger pathways, including protein kinase C (Hammond et al. 2004; Crepel et al. 2003). However, the potentiation of synaptic transmission in anoxic LTP has been reported to be mediated by both post-synaptic NMDA and AMPA receptors (Krnjević 2008). These observations indicate that ischemic-like episodes in vitro can result in strengthened excitatory synaptic transmission through LTP-like mechanisms. This is predicted to result in hyperexcitability, which, as discussed above, is believed to increase the probability of seizure.
genesis. In line with this viewpoint, glutamate exposure can result in epileptiform activity in organotypic slice cultures (Sun et al. 2001).

Alternatively, Hartings et al (2003) hypothesized that neuronal depolarization induced by high extracellular potassium ions (K$^+$) may be responsible for triggering seizures. Anoxic/ischemic depolarization occurs in the acute phase of an ischemic/anoxic insult during which K$^+$ can increase up to 60 mM (Nedergaard and Hansen; 1992; Somjen 2001). An increase in extracellular K$^+$ (more precisely, the ratio of extracellular to intracellular potassium concentrations) can have complex effects on synaptic transmission depending on the concentration. Mild increases can result in both an enhancement of the amount of neurotransmitter released from presynaptic terminals and an increase in post-synaptic excitability (Somjen 2002; Somjen 2004), which, in turn, are expected to increase neuronal excitability. Alternatively, increased extracellular potassium may inactivate voltage-gated channels; this is sometimes referred to as ‘depolarization block’ (Somjen 2002; Somjen 2004). If this occurs in principal cells, cellular excitability will decrease, precluding seizure genesis; however, if it occurs in inhibitory interneurons, it may relieve inhibition, which can indirectly increase excitability of principal cells. Indeed, the combination of hypoxia and artificially elevated extracellular K$^+$ (from 2.5 to 8.5 mM) resulted in epileptiform discharges in acutely prepared hippocampal slices in vitro (Kawasaki et al. 1990), while increases in extracellular K$^+$ are associated with epileptiform activity in disinhibited brain slices in vitro (Borck and Jefferys. 1999)

Both glutamate and K$^+$ increase during cortical spreading depression, which is a propagating wave of depolarization implicated in the pathogenesis of stroke, migraine, and traumatic brain injury (Somjen 2001; Lauritzen et al. 2011). In the context of ischemia it is commonly referred to as anoxic depolarization, hypoxic spreading depression, ischemic depolarization, or terminal depolarization. Spreading depression-like events have been observed during the acute phase of an ischemic episode and can recur throughout the ischemic penumbra in humans and animal models (Strong and Dardis 2005; Hopwwod et al. 2008; Lauritzen et al. 2011). The latter events are referred to as peri-infarct depolarizations and are believed to expand ischemic brain injury. Interestingly, employing electrocorticography Fabricius et al (2008) noted a co-occurrence of electrographic seizures and cortical spreading depression in human patients with severe brain injury. This observation suggests that the mechanisms of post-ischemic seizure genesis may be
associated with the mechanisms of cortical spreading depression in the injured brain; however, a clear understanding of this relationship remains to be elucidated.

Interestingly, there is evidence that direct exposure of brain cells to blood constituents such as albumin and thrombin can result in seizures (Kelly 2008). In the context of stroke, this may occur during hemorrhage or breakdown of the blood brain barrier (Friedman et al. 2009b; del Zoppo 2010). Notably, intracerebral injection of thrombin resulted in motor seizures in rats (Lee et al. 1997). Moreover, the application of thrombin to rodent hippocampal brain slices results in the potentiation of the glutamatergic transmission in the CA1 region, and also in a reduced threshold to the development of epileptiform activity in response to elevated extracellular glutamate and potassium ions (Maggio et al. 2008). Thrombin is a serine protease that converts fibrinogen to fibrin in the blood coagulation cascade. Though the mechanism for its hyperexcitable effect is unknown, it seems to be due to a direct action of thrombin on protease activated receptors (PARs), which are involved in hemostasis, thrombosis, and inflammation (Maggio et al. 2008).

Although increased excitability as consequence of factors such as ionic disturbances, anoxic LTP, and exposure to blood constituents may explain the predisposition of the ischemic brain to seizures, it is still unclear precisely how and where such alterations actually trigger seizures. Furthermore, given the close association of these factors to excitotoxicity and neural injury, it is difficult in practice to examine their putative ‘pro-seizure’ effects independently from their roles in ischemic brain injury in in vivo experimental models of stroke. The latter point is particularly relevant when interpreting the effects of anticonvulsant drugs in animal models of post-ischemic seizures, which may themselves be neuroprotective independent of their ability to inhibit seizures.

1.7 Post-ischemic seizures and brain injury

It has been suggested that early-onset seizures may exacerbate ischemic brain injury by placing an added metabolic burden on an already metabolically compromised hypoperfused brain (Williams et al. 2004; Williams et al. 2006; Friedman et al. 2009a; Burneo et al. 2010). Accordingly, anticonvulsant drugs inhibit post-ischemic seizures, reduce lesion size, and improve survival in rats and mice; albeit, these treatments are more efficient when administered prophylactically (Williams et al. 2004; Williams et al. 2006).
Seizures, particularly during status epilepticus, have long been suspected to result in brain injury \textit{per se} - not exclusively in the context of cerebral ischemia. The exact mechanisms of how seizures result in brain injury have not been completely resolved. Seizures may result in brain injury through at least three proposed mechanisms: 1) Over-activation of glutamate receptors during recurrent seizure activity may result in excitotoxicity, similar to that proposed in cerebral ischemia. 2) Seizures may place an increased metabolic demand on neural tissue, and if this persists, it may lead to metabolic failure and cell death. 3) Seizures may result in altered cerebral blood flow dynamics (Somjen 2004). It is a well-established observation that seizures can alter the dynamics of cerebral blood flow. Seizures can increase cerebral blood flow to involved brain regions (this presumably reflects the coupling of synaptic activity to cerebral blood flow); however, if seizure activity persists, as in status epilepticus, then blood flow to the involved region can actually be reduced through currently unknown mechanisms (Siesjo and Wieloch 1986; Lothman 1990; Somjen 2004; Fabene et al. 2007). This has recently been confirmed with sensitive magnetic resonance imaging and arterial spin-labeling techniques in rats, and these studies have demonstrated a correlation between decreased blood flow in brain regions undergoing continuous ictal discharges and subsequent neuronal injury (Engelhorn et al. 2005; Schriddle et al. 2008; Choy et al. 2010a; Choy et al. 2010b). Any of the above three mechanisms could, in principle, contribute to brain injury following cerebral ischemia. Thus, seizures may in fact expand ischemic injury and lesion size, though direct experimental support for this hypothesis in the context of early onset post-stroke seizures is generally lacking.

1.8 Generalized early-onset post-stroke seizures summarized

In summary, animal models of stroke can result in post-ischemic seizures in adult rats. However, the reported incidence of post-ischemic seizures in rodent models varies from study to study. Interestingly, as described in humans (see 1.4), the behavioral manifestations of these seizures are also variably documented; specifically, reports range from no behavioral abnormalities in some studies to outright convulsions in others. This is a curious observation, and it remains to be determined how cerebral ischemia can result in both convulsive and non-convulsive seizures, and whether these two phenomena arise through similar cellular mechanisms or represent distinct subsets of post-ischemic seizures. Notably, a clear documentation of the EEG alterations associated with post-ischemic seizures is currently available only for non-convulsive seizures following the filament middle cerebral artery occlusion (MCAO) model in rats; whether these
EEG characteristics extend to convulsive post-ischemic seizures is currently unknown. Likewise, anticonvulsants have demonstrated utility in treating only the non-convulsive seizures following the filament MCAO model in rats; whether these treatment regimens extend to convulsive seizures remains to be determined. Moreover, a convincing association of early-onset seizures, convulsive or not, with mortality and brain injury is, in my opinion, currently incomplete. Finally, the mechanisms of seizure genesis, convulsive or not, are currently unclear.
Chapter 2

2 Experimental model and hypotheses

2.1 Hypoxia-ischemia as a model of large hemispheric stroke

To date, several rodent models of cerebral ischemia have been developed and extensively characterized (Hossmann 2007; Durukan and Tatlisumak 2009; Howells et al. 2010). In laboratory parlance, models of cerebral ischemia are referred to as either ‘focal’ or ‘global’ models. Focal models of cerebral ischemia involve the reduction of cerebral blood flow to a localized brain region, typically achieved through the experimental occlusion of one of the major cerebral arteries or its branches. Approximately two-thirds of ischemic strokes in humans occur in the territory of the middle cerebral artery, and this has spawned the development of a family of focal middle cerebral artery occlusion (MCAO) models in rodents. These include the highly invasive direct surgical occlusions such as the transorbital and transcranial MCAO, the less invasive intraluminal filament MCAO, a number of thromboembolic MCAO models, and endothelin-1-induced MCAO. Focal MCAO models result in infarction in the territory of the middle cerebral artery within a few hours of the occlusion, with a primary region of damage to the ipsilateral parietal cortices and the basal ganglia. Other focal models targeting smaller arteries have been developed, including occlusion of the cortical pial vessels and microvasculature by methods such as photothermbotic occlusion and a number of microembolic models. Models of spontaneous infarction also exist such as the stroke-prone spontaneously hypertensive rats (SHR-SP) that exhibit spontaneously occurring brain infarcts. A number of venous occlusions also exist but rarely are used.

On the other hand, global models of cerebral ischemia involve the reduction of blood flow to large portions of the brain, and include, for example, experimentally-induced cardiac arrest and bilateral occlusion of the common carotid artery (Hossmann 2007; Epsztein et al. 2008). The latter is sometimes combined with systemic hypotension or occlusion of the vertebral or basilar arteries. Global ischemia models are usually carried out transiently, and typically result in minor, delayed, and specific damage usually restricted to the hippocampal CA1 sector, layers 3, 5, and 6 of the cerebral cortex, and the striatum (Hossmann 2007).
In addition, there are a number of experimental models of cerebral ischemia that are arguably intermediate between focal and global ischemia. For example, in gerbils and in certain strains of mice, unilateral occlusion of the common carotid artery results in multi-focal ipsilateral hemispheric brain injury (Hossmann 2007). In rats and C57BL/6 mice\(^4\), unilateral occlusion of the common carotid artery does not result in brain injury, and is therefore combined with respiratory hypoxia (Hossmann 2007). The latter model is referred to as hypoxia-ischemia (HI).

These experimental models have vastly improved our understanding of the complex molecular-biochemical, structural, neurophysiological, and behavioral alterations that occur as pathological consequences of cerebral ischemia.

I have employed the model referred to as hypoxia/ischemia (HI) in adult male C57BL/6 mice. This model was initially developed in rats (Levine 1960), and later modified and extended to adult mice (Vannucci et al. 2001). The procedure consists of a permanent unilateral ligation of the common carotid artery under isoflurane anesthesia and subsequent respiratory hypoxia (8% O\(_2\) for ~30 minutes). Notably, the hypoxic episode can be carried out in either anesthetized or non-anesthetized mice with similar patterns of subsequent brain injury (Adhami et al. 2006). The HI model is amenable to investigating cerebral ischemia in small rodents due to its minimal surgical invasiveness, sparing use of anesthetics, and suitability in age-advanced and various transgenic mice (Vannucci et al. 2001; Héron-Milhavet et al. 2004; Olson and McKeon 2004; Olson et al. 2004; Wang et al. 2009).

The histological, molecular, and behavioral consequences of HI in adult mice have been well documented. HI results in a reduction in ipsilateral cerebral blood flow (Vannucci et al. 2001; Adhami et al. 2006), breakdown of the blood brain barrier and cerebral edema (Adhami et al. 2006), cortical and subcortical infarction throughout the ipsilateral hemisphere within 6 hours of the HI episode (Vannucci et al. 2001; O'Donnell et al. 2002; Gilbert et al. 2003; Héron-Milhavet et al. 2004; Olson and McKeon 2004; Zhang et al. 2004; Adhami et al. 2006; Wang et al. 2009), with the development of penumbral-like zones (Olson and McKeon 2004), apoptotic and

\(^4\) The Circle of Willis is incomplete in these animals, resulting in compromised collateral blood flow during unilateral occlusion of the common carotid artery. In rats and C57BL/6 mice, the Circle of Willis is relatively better developed, and unilateral occlusion of the common carotid artery does not result in appreciable brain injury due to sufficient collateral flow.
necrotic cell death (Olson and McKeon 2004; Adhami et al. 2006), microglial and astrocytic activation and increased expression of pro-inflammatory cytokines (Olson and McKeon 2004; Zhang et al. 2004), alterations in gene expression (Gilbert et al. 2003), motor deficits (Olson and McKeon 2004), and mortality (Vannucci et al. 2001).

One particular concern about the HI model is the use of an artificial hypoxic episode. This may have complex systemic effects that influence brain injury in this experimental model. Three studies have attempted to address this issue (Vannucci et al. 2001; Fowler et al. 2003; Adhami et al. 2006), and have examined a number of systemic parameters, including respiration rate (Adhami et al. 2006), heart rate (Vannucci et al. 2001; Fowler et al. 2003; Adhami et al. 2006), mean arterial blood pressure (Vannucci et al. 2001; Fowler et al. 2003; Adhami et al. 2006), partial pressures of oxygen and carbon dioxide in arterial blood (Vannucci et al. 2001; Adhami et al. 2006), blood pH (Vannucci et al. 2001; Adhami et al. 2006), and blood and brain glucose and lactate levels (Vannucci et al. 2001). Although these authors noted transient and reversible alterations in the above-mentioned parameters during the hypoxic episode, they concluded that HI primarily results in ipsilateral brain injury through a severe reduction in ipsilateral cerebral blood flow.

Notably, Adhami et al (2006) have suggested that the pattern and course of brain injury following HI resembles the clinical manifestations of large hemispheric, complete carotid territory, or panhemispheric strokes, which are relatively rare but dangerous and severe forms of ischemic stroke in humans⁵ (Schwarz et al. 2001; Steiner et al. 2001; Kumaral et al. 2009). Additionally, HI is believed to be particularly relevant to clinical conditions that can result in both diminished cerebral blood flow and reduced oxygen tension, such as during coronary bypass or neurovascular surgeries (Olson and McKeon 2004).

However, despite the extensive use and characterization of HI as a model of focal cerebral ischemia, an in depth electrophysiological assessment of HI in adult mice is lacking. In

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⁵ The exact incidences of these types of stroke are currently unclear, but estimates range from 5-10% of ischemic stroke cases (Schwarz et al. 2001). The most common cause is cardioembolic occlusion of the internal carotid artery in conjunction with poor collateral flow through the Circle of Willis. In the most severe cases, this can result in catastrophic panhemispheric infarction across large portions of the ipsilateral hemisphere, including regions supplied by the middle, anterior, and posterior cerebral arteries.
particular, electroencephalographic (EEG) activity has demonstrated utility in assessing ischemic injury in human subjects (Jordan 2004; Finigan et al. 2007; Sheorajpanday et al. 2009) and animal models (Raffin et al, 1991; Lu et al. 2001; Williams et al. 2001; Braida et al, 2003; Lee et al, 2005; Tichauer et al, 2009), yet an examination of EEG activity following HI in adult mice has not been carried out.

More importantly, the possibility of early-onset post-ischemic seizures following HI in adult mice has not been explored. HI in adult mice may be suitable for the examination of early post-ischemic seizures for three main reasons: 1) Compared to other ischemic models, it is a minimally invasive procedure that does not require extensive anesthesia. This may reduce potential confounding effects of anesthetics and long, complex surgeries on recovery time and seizure genesis. 2) It is worth noting that sham-operated rats in MCAO studies (control animals in studies of middle cerebral artery occlusion) can develop spontaneous non-convulsive seizures (Karhunen et al. 2005; Kelly et al. 2007). The use of mice instead of rats and a simple protocol to induce ischemia may circumvent this potential confounding complication. 3) HI results in a consistently large and extensive area of brain injury. As discussed above (see 1.4), this has been correlated to an increased risk of post-stroke seizures in humans.

2.2 Aims and hypotheses

2.2.1 General aims

The general aims of this study are:

1) To determine whether HI in adult mice can serve as a valid model to study early-onset post-stroke seizures.

2) To determine the behavioral and neurophysiological characteristics of post-HI seizures and their role in influencing post-HI outcome.

2.2.2 Specific hypotheses

1) HI will result in extensive brain injury throughout the ipsilateral (relative to the carotid occlusion) cerebral hemisphere of adult male C57BL/6 mice under my experimental conditions.
2) HI will result in early-onset seizures in adult male C57BL/6 mice. These seizures may be convulsive or non-convulsive.

3) The extent of ischemic brain injury is a significant risk factor for the development of post-HI early-onset seizures.

4) Post-HI seizures will be associated with a significantly increased risk for mortality.

5) Treatments with anticonvulsant drugs will effectively reduce: a) seizure incidence, b) brain injury, and c) mortality.

6) EEG activity will:
   a) Be extremely suppressed in post-HI animals, and the magnitude of suppression will be significantly associated with brain injury, mortality, and the development of post-HI seizures.
   b) Demonstrate clear electrographic ictal discharges in ipsilateral brain regions with or without associated motor convulsions.
3 Methods

3.1 Animals

Male C57BL/6 mice (Charles River Laboratory, Quebec, Canada) at ages of 4-9 months were used as the goal here is to model ischemic brain injury and post-ischemic seizures in adult animals. The age-range is due to the commercial availability and age of retired breeder mice. Mice of 4-9 months may roughly correspond to a human age-range of 20 to 40 years (Flurkey et al. 2007). Although the age-range is arguably wide, no obvious effects of age were noted in this study; furthermore, all control animals in this study were age-matched. The animals were kept in a vivarium that was maintained at 22ºC–23ºC and had a 12:12 hour light:dark cycle. Food and water were available ad libitum. All experiments conducted in this study have been reviewed and approved by the animal care committee of the University Health Network. Efforts were taken to minimize pain and the number of animals required, and to adhere to the suggested guidelines for preclinical stroke studies (STAIR 1999; Macleod et al. 2009).

3.2 Hypoxia-Ischemia (HI)

HI was conducted as per previous studies (Vannucci et al. 2001; Olson and McKeon 2004). Mice were operated under isoflurane anesthesia (2%). The right common carotid artery (RCCA) was isolated and ligated with fine surgical thread (Softsilk, 5-0, United States Surgical, Norwalk, Connecticut, USA). Care was taken to avoid damage to the vagus nerve and other blood vessels. The surgery was typically completed within 10 minutes. Hypoxic episodes were conducted 1.5 hours after RCCA ligation (unless otherwise indicated), at which point the animals had recovered from anesthesia and exhibited ‘normal’ behaviors, such as exploring and grooming. An airtight plastic chamber (modular incubation chamber, Billups-Rothenberg, Del Mar, CA, USA; Wais et al. 2009) was used to conduct the hypoxic episode (8% O₂–92% N₂ for ~30 min). The chamber has two (input and output) air paths and a total volume of 5.89L. A small hole was made at the top of the chamber, allowing soft wires to pass through and connect to the implanted electrodes. A rubber gasket was used to seal the hole to prevent air leakage. During the hypoxic episode, the
chamber was continually flushed with 8% O₂–92% N₂ at a flow rate of 15 L/min. As per the manufacturer’s instructions, equilibration is achieved in 6 minutes at this flow rate. Inner air temperature of the chamber was monitored continuously via a fine thermo probe (Digi-Sense, Cole Parmer), and the temperature was maintained at 35-35.5 ºC (Vannucci et al. 2001) via heating pads placed beneath the chamber. Sham control mice received RCCA isolation but not ligation, and were exposed to similar conditions except the chamber was flushed with room air.

After HI, the animals were allowed free access to food and water. Mandatory euthanization was conducted in compliance with ethical guidelines if animals exhibited long-lasting immobility together with irresponsiveness to lateral push, and a lack of eating and drinking, which were almost always associated with unsubsiding, severe convulsions. Sham operation (n=23 mice) or RCCA ligation alone (n=14 mice; see Appendix II) did not result in mortality, seizures, or mandatory euthanization.

3.3 Behavioral seizure monitoring and treatment

The majority of animals were monitored visually for the first 6 hours and again at 48 and 72 hours following HI to detect the occurrence of severe (Stages 4-5) behavioral seizures described below. Additionally, 45 animals were continuously monitored via video recordings for the first 24 hours following HI for detailed analysis of convulsive behavior. Furthermore, 14 animals (3 with early severe seizures) underwent 24-hour video monitoring at later post-HI times (2-4 times over 1-4 weeks) to detect possible late-onset seizures. The behavioral (motor) seizures were determined using a modification of a 0-5 scale established for generalized seizures (Velíšková 2006). Stage 0 or 0.5 refers to no abnormality or minor abnormalities such as excessive sniffing or grooming. Stage 1 or 2 refers to isolated myoclonic jerks or atypical clonic seizing. Stage 3 represents fully developed bilateral forelimb clonus, including tonic components and body twists. Stage 4 corresponds to fully developed tonic-clonic convulsions. Stage 5 refers to stage 4 plus running-bouncing clonic fits. In all cases of severe seizures, ≥ 2 episodes of stage 4-5 seizures were observed for individual animals.

A group of post-HI animals were treated with a combination of diazepam (0.15mg/Kg) and phenytoin (18mg/Kg). A clinically available injectable form of these drugs approved for human usage (Sandoz Canada Inc; Quebec, Canada) was mixed with saline immediately before use and administered by a bolus intra-peritoneal injection (10ml/kg) either within 5 minutes after
termination of the hypoxic episode (n=31 mice) or at the onset of the first observed motor seizure (within 3 hours post-HI; n=8 mice). Control animals received saline injections (n=16 mice). All treated animals were monitored by video recordings for the first 24 hours post-HI, and visually for 6 hours at 48 and 72 hours post-HI. Seizure assessments were carried out by an experimenter blind to animal information.

3.4 Intracranial electroencephalographic (EEG) recordings

3.4.1 Surgery and electrode implantation

Electrode implantation, data acquisition and analysis were conducted as previously described (Wu et al. 2008; Wais et al. 2009; D’Cruz et al. 2010). Mice were anesthetized under isoflurane anesthesia (2%) and secured in a stereotaxic frame. A skin incision was made, and three small holes drilled through the skull. A pre-constructed electrode assembly was inserted into the brain and secured in place with a cyanoacrylate-based glue (Insta-cure+, BSI Adhesives, Atascadero, CA, USA). The electrodes were made with polyimide-insulated stainless steel wires (outside diameter 0.125 mm, Plastics One, Roanoke, VA, USA), and implanted into the right (ipsilateral to RCCA ligation) dorsal hippocampal CA1 area (bregma −2.3 mm, lateral 2.0 mm and depth 2.0 mm) and contralateral somatosensory cortex (bregma −0.6 mm, lateral 1.5 mm and depth 1 mm). A reference electrode was placed superficially into the ipsilateral motor cortex (bregma +1.2 mm, lateral 2.0 mm, depth 0.5 mm). This recording montage has permitted a reliable detection of EEG ictal discharges in juvenile mice following hypoxic challenges (Wais et al. 2009; Figure 2) and in adult rats following intra-hippocampal injections of cobalt (He et al. 2009). In some experiments, recording electrodes were implanted bilaterally in the hippocampal CA1 regions or in entorhinal cortical regions (bregma −4.8 mm, lateral 2.8 mm and depth 2.5 mm). In telemetry recordings (see below), only the right (ipsilateral to RCCA ligation) hippocampal CA1 or parietal cortex region was recorded. Recordings were verified via histological assessments and EEG characteristics. The tracks of EEG electrodes were verified in cresyl violet-stained brain sections of 17 mice (see Figure 12).
Figure 2. Hypoxia-induced seizures in juvenile mice
**Figure 2. Hypoxia-induced seizures in juvenile mice**

The method of intracranial EEG recordings employed in this study has previously demonstrated efficacy in detecting ictal EEG discharges following global hypoxia in juvenile mice. 

**A**, Raw EEG traces acquired from a juvenile mouse one week after being implanted with intracranial electrodes in right hippocampus (CA1 region) and left somatosensory cortex. 

**B**, The mouse was then subjected to a global hypoxic episode (4% O₂-96% N₂, ~30 mins). Note that EEG activity was suppressed in both channels during the hypoxic episode. 

**C and D**, Within 15 mins following return to normoxia, generalized ictal EEG activity was observed in both channels. Ictal discharges featured sequential spikes and poly-spike runs. These discharges exhibited gradually increasing amplitudes up to 5 mV and then terminated with post-ictal suppression phases of 10–20s. In association with such ictal discharges, the animals showed evidence of convulsive motor activity, including tail erection, frequent head and forelimb movements and/or rearing. 

**E and F**, EEG activity at 30 mins and 22 days following hypoxia. Note a recovery in EEG amplitude. 

Figure modified from Wais et al (2009) with permission from Elsevier.
3.4.2 Quantification of EEG activity

Baseline EEG activities were recorded after ≥ 7 days of recovery from implantation. Recordings were performed during and following HI and intermittently in 2-hour sessions at later post-HI times. Telemetry EEG recordings (see below) were performed continuously for up to 72 hours post-HI. Extracellular amplifiers with extended head-stages (Model-3000, AM Systems Inc., Carlsborg, WA, USA) were used, and EEG activities were recorded in a frequency band of 0.05–1000 Hz and amplified 1000 times before digitization (digitization rates of 60 kHz, Digidata, 1300, Molecular Devices, Unit City, CA, USA). Data acquisition, storage and analyses were done using pCLAMP software (version 10, Molecular Devices). Sampling rate was digitally reduced by a factor of 10 prior to analysis.

To quantify changes in EEG suppression, mean amplitude, variance, and root mean square (RMS) were calculated from 30-second long EEG segments. For the calculation of EEG amplitude, variance, and RMS over time, EEG activity was analyzed only during periods of immobility for three reasons. 1) EEG activity is more stable and larger in amplitude during periods of extended immobility. 2) It is also less prone to movement-related artifacts. Movement artifacts were defined as sudden large-amplitude distortions (beyond the range of the digitizer) above background EEG activity that were temporally correlated to any strong bending of the soft recording wires, as would occur when an animal pulled on the wires during grooming or running. 3) Animals were generally immobile during and shortly after the hypoxic episode; thus, in order to maintain consistency and to carry out analyses of EEG amplitude over time (including the hypoxic episode), periods of immobility were analyzed for these parameters. EEG segments for analysis were selected according to the following criteria: 1) The animal had been allowed at least 1 hour to become accustomed to the recording environment. 2) Clear slow-wave activity in the cortical recordings was visible. 3) Segments were flanked by at least 30 seconds of near continuous immobility.

Mean EEG amplitude and variance were calculated from digital EEG signals using the histogram function of pClamp software. A histogram was generated from a continuous 30-second EEG data segment with a bin-width of 0.01mV, each bin being associated with a count consisting of the number of data points within that bin. Histogram counts per bin were transferred to SigmaStat software (11th version, Systat Software Inc, San Jose, California, USA) and re-expressed as
probabilities by dividing individual counts per bin by total count. Mean amplitude, rectified mean amplitude, and variance were calculated according to standard formulae for discrete random variables.

Mean amplitude was calculated as \( \sum vp \) where \( v=\text{mV (bin)} \) and \( p=\text{probability} \). This mean was generally close to zero (the EEG signal is generally symmetrical around 0mV) and was used to calculate variance but is not reported in the thesis.

Variance was calculated as \( \sum (v-\text{mean amplitude})^2p \).

Rectified mean amplitude was used to examine EEG alterations in this thesis and was calculated as mean amplitude above except that histogram bins were rectified to display only positive EEG voltages.

EEG root mean square was calculated from a continuous 30-second EEG data segment in pClamp. Power spectra were generated by averaging 10 spectral segments with 50\% window overlap and a spectral resolution of 0.407 Hz (D’Cruz et al. 2010). The root mean square (in mV) was calculated in pClamp by taking the square root of the integral of the power spectrum.

For quantification of hippocampal theta rhythms, 4-second EEG segments during continuous movement were collected (8-10 segments per animal; D’Cruz et al. 2010). Power spectra were generated with a spectral resolution of 0.407 Hz, and dominant frequency (in Hz) and power (mV\(^2\)/Hz) were obtained and averaged for each animal at indicated time-points.

For spectral analyses across all frequency bands, power spectra were generated from continuous 30-second EEG data segments as described above for root mean square analysis. Dominant frequency and power were obtained at frequency bands of approximately 1-4Hz (delta), 4-12Hz (theta), 12-30Hz (beta), 30-100Hz (gamma), and 100-500Hz (high frequency, HF). These frequency bands were chosen in accordance with the known dominant frequency bands of the hippocampal EEG signal (O'Keefe 2007).

### 3.5 Telemetry recording

A data acquisition system (Dataquest A.R.T.) with mouse-specific transmitters (TA11ETA-F10, Data Science International, St. Paul, MN, USA) was used for recording body temperature and
electrocardiogram (ECG) or EEG. The surgical procedures were modified from Weiergräber et al (2005). Briefly, the animal was anaesthetized with 2% isoflurane and the ventral abdominal wall was opened to place the transmitter (including a thermosensor) in the abdominal cavity. For EEG, sensing and reference wires connecting the transmitter were orientated rostrally towards the head via a subcutaneous route. The sensing wire was soldered to an intracranial EEG electrode as described above and the reference wire was placed caudally and epidurally at Bregma -5 mm and lateral 1 mm (Weiergräber et al. 2005). For ECG, the sensing wire was sutured onto the abdominal wall with an exposed piece in close contact with muscles. The reference wire was placed subcutaneously near the neck. To prevent potential infection, animals were treated with the antibiotic enrofloxacine (Baytril, Bayer HealthCare, Ontario, Canada), which was administered orally via drinking water at an estimated dose of 0.7 mg per day, 3 days before and 5 days after the surgery. To relieve post-surgery pain, animals received a subcutaneous injection of the analgesic metacam (Boehringer Ingelheim, Burlington, Ontario, Canada; 4mg/Kg). The surgery and transmitter implantation caused no evident abnormality in animal behavior. Measured immediately and 10 days post surgery, animal body weights were 29.4±0.7g and 29.2±0.4g respectively (n=11 mice). Measured 10 days post surgery and before HI, body temperatures were 37.0±0.22ºC and heart rates were 459.6±51.9 beats/min. Overall, these observations are in keeping with previous telemetry studies in mice (Weiergräber et al. 2005; Arraj and Lemmer 2006; Ovechkin et al. 2006).

HI was conducted as described above except the hypoxic chamber was placed onto a telemetry receiver. Body temperature and EEG or ECG were recorded continuously during and following the hypoxic episode. Averages from 3-min recording sessions were calculated for baseline assessments and at different times post-HI.

3.6 Doppler measurements of cerebral blood flow

A laser Doppler system (PF5010) with mouse-specific probes (MTB 500, tip diameter of 0.5mm, Perimed, Järfälla, Sweden) was used to measure local cortical blood flow through the skull. These experiments were conducted in anesthetized mice (2% isoflurane) (Adhami et al. 2006). After isolating and surrounding the RCCA loosely with a suture, the animal was positioned into a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA) with ear bars and a frontal teeth-hook. A custom mouth-mask was used to keep the animal anesthetized. Following a
skin incision and skull exposure, a MTB probe was positioned onto the skull surface via a micromanipulator. The probe’s location was adjusted to ensure a stable and relatively high baseline measurement (perfusion units). The stereotaxic coordinates of probe locations were Bregma 2.67±0.21 mm and lateral 1.92±0.11 mm (n=15 mice). Under these conditions, blood flow measurements were insensitive to movement-related artifacts. During the measurement, the animal was warmed by a heating bag, and its rectal temperature was maintained at 37-37.5°C. After baseline measurements, HI was carried out by tying the pre-positioned suture followed 5 minutes later by changing the isoflurane-delivery air from 100% O2 to 8% O2 -92% N2. Blood flow (perfusion units) was sampled continuously every 1 second. Averages from 3-min segments were calculated from individual animals at baseline and at different times post-HI and data were normalized as percent of baseline blood flow.

### 3.7 Histology

Histological assessments of brain tissues were conducted as previously described (Wu et al. 2005; Wais et al. 2009). Briefly, mice were anesthetized with an intra-peritoneal injection of sodium pentobarbital (70 mg/Kg, Somnotol, WTC Pharmaceuticals, Ontario, Canada) and transcardially perfused with a 10% neutral buffered formalin solution. The brain was removed, and a series of coronal sections were obtained at 8 or 50 μm thickness and stained with cresyl violet (Nissl stain). The use of 50 μm sections was for better preservation of injured brain tissue during sectioning and for convenience in verifying EEG electrode tracks. Stained sections were photographed under a dissecting microscope, and measurements of infarct area were conducted using Image J software (National Institute of Health, USA). Quantification of infarct area was adapted from Adhami et al (2006). An experimenter blind to individual animal information was asked to assess whether infarction was clearly evident in large regions of one hemisphere that included the hippocampus, cerebral cortex, and thalamus. Only animals exhibiting clear ipsilateral brain injury were chosen for analysis. Infarct area was estimated at 8 coronal planes corresponding to Bregma -3.2 mm, -2.4 mm, -1.5 mm, -1.1 mm, -0.2 mm, 0.5 mm, 1.2 mm and 1.9 mm (Franklin and Paxinos 1997). Ipsilateral infarction was expressed as the percent reduction in total ipsilateral relative to total contralateral hemispheric area at each plane.

TUNEL (Millipore corporation, Toronto, Ontario, Canada) and FluoroJade C (Histo-Chem, Inc., Jefferson, Arkansas, USA) staining was carried out on 20 μm fixed sections according to the
manufacturer’s instructions and protocols established in previous studies (Austin and Fehlings 2008; Wais et al. 2009; Yu et al. 2009). TUNEL sections were co-stained with DAPI (Millipore) to verify nuclear localization of TUNEL-positive signals (data not shown). For quantification of fluorescent signals, images were acquired with a 20x objective, resulting in an imaging field of 0.13mm$^2$. The imaging field was restricted to regions of the temporal cortex (perirhinal and entorhinal areas) because of the high vulnerability of these regions in this hypoxia-ischemia model. Analyses of all data were carried out by an experimenter blind to individual animal information. In the cases of animals with minimal/unclear brain injury, it is likely that bias was introduced by the experimenter in selecting imaging fields, such that these signals were over-estimated in these animals (see Figures 5 and 6). Signal counting and image processing was carried out with Image J software. Counts from three brain sections were averaged per animal and were expressed as total count per mm$^2$.

3.8 In vitro electrophysiology

The procedures for preparing brain slices were modified as per previous studies (Wais et al. 2009; Wu et al. 2005). Briefly, the animals were anesthetized by an intra-peritoneal injection of sodium pentobarbital (70 mg/Kg, Somnotol, WTC Pharmaceuticals, Cambridge, Ontario, Canada), and underwent a trans-cardiac infusion with a cold (4ºC), low-Na$^+$/Ca$^{2+}$ artificial cerebrospinal fluid (ACSF) before decapitation. The brain was hemisected, and the brainstem tissue was removed to extend the curved hippocampus. The remaining brain tissue was glued onto an aluminium block with the longitudinal axis of the hippocampus being perpendicular to the horizontal cutting plane. Slices of 0.5 mm thickness were obtained in ice-cold, low-Na$^+$/Ca$^{2+}$ ACSF using a Vibratome. After vibratome sectioning, the slices were stabilized in standard ACSF at 35ºC for 30 minutes. During the stabilization period, 2 mM kynurenic acid, a general antagonist for ionotropic glutamate receptors (Research Biochemicals Inc./Sigma-Aldrich, Mississauga, Ontario, Canada), was included in the ACSF to prevent potential post-dissection glutamate toxicity. After the 30-minute stabilization, slices were rinsed and then maintained in the standard ACSF at 22ºC for 1-6 hours before recording. The low-Na$^+$/Ca$^{2+}$ ACSF contained (in mM): NaCl 50, choline chloride 80, KCl 3.5, NaH$_2$PO$_4$ 2, CaCl$_2$ 0.5, MgCl$_2$ 7, glucose 20 and HEPES 5 (pH adjusted to 7.4). The standard ACSF contained (in mM): NaCl 125, KCl 3.5, NaH$_2$PO$_4$ 1.25, NaHCO$_3$ 25, CaCl$_2$ 2, MgSO$_4$ 1.3 and glucose 10 (pH of 7.4 when aerated with 95%O$_2$-5%CO$_2$).
Extracellular recordings were conducted in a submerged chamber at 35°C (Wu et al. 2005). Extracellular recording electrodes were made with thin-wall glass tubes (1.5 mm outside diameter, World Precision Instruments, Sarasota, Florida, USA). These electrodes were filled with a solution containing 150 mM NaCl and 2 mM HEPES (pH 7.4) and their resistance was 1~2 MΩ. A bipolar electrode made of the polyimide-insulated stainless steel wires (see above) was used for afferent stimulation. Constant current pulses (0.1 ms duration, 20-150 μA) were generated by a Grass stimulator (S88) and delivered through an isolation unit every 30 seconds. The stimulating electrode was placed in the CA2 stratum radiatum area to stimulate the Schaffer collateral pathway, and field EPSPs were recorded from the CA1 apical dendritic layer. Data acquisition, storage and analysis were done using the pCLAMP package (version 10).

3.9 Statistical analyses

Statistical tests were conducted via SigmaStat software (11th version, Systat Software Inc, San Jose, California, USA).

For a direct comparison of two groups, a Student’s t test or Mann-Whitney Rank Sum Test was carried out.

For comparing proportions, a Fischer Exact Test was employed.

For single group comparisons, a one-way ANOVA was used. For multiple group comparisons over time, a general linear model (GLM) two-way ANOVA was employed. If significance was observed, this was followed by a Student-Newman-Keuls multiple comparison pairwise test.

When possible, raw data values were employed for statistical analysis. However, in most cases data was expressed as percent of baseline. This was carried out for three reasons: 1) To simplify data display and presentation. 2) To reduce variability in cases where repeated measures testing was not ideal (i.e., mortality). 3) To make data better fit with the assumptions of normality and equal variance required by some statistical tests (i.e., the two-way ANOVA, for which there is no non-parametric alternative). In some cases, a square root transformation was applied to make data fit to the assumptions of normality.

The minimum level of significance was set at α=0.05. Exact p-values are reported in the text, except for data analyzed on ranks (in this case p-values can only be reported as < or > 0.05).
Moreover, p-values less than 0.001 are not reported precisely but are indicated as ‘p<0.001’ (this is the lowest p-value that is reported by SigmaStat). Adequate statistical power (>70%) was noted in the majority of tests. If a negative result (p>0.05) was coupled with low power, the possibility of a false-negative was mentioned as a caveat in the text.

In general, only significant differences across groups are reported in the text. For example, significant group differences in mean EEG amplitude are reported between animals exhibiting extensive brain injury, animals with minimal injury and sham controls at multiple time-points. In general, statistically significant differences within individual groups are not presented e.g. mean EEG amplitude only in animals with brain injury relative to baseline. This was carried out for four reasons: 1) To simplify data display and presentation. 2) To address the difficulties associated with the two-way ANOVA described above. 3) Differences across groups generally mirrored differences within groups, and any discrepancies are reported. 4) No significant differences were observed within sham control groups.

Mean and standard error of the mean are presented throughout the text and figures.
Chapter 4

I have organized the results into six overlapping chapters numbered 4 through 9. The chapters are ordered in a manner such that key concepts and terminology are developed early on, and these concepts are expanded upon and utilized as foundations in later sections. Each section opens with a brief introductory statement, which is followed by further sub-sectioning, and is concluded with a summary statement. To facilitate data interpretation, I have also drawn attention to important caveats and conclusions that will be addressed in greater detail in the discussion.

The results open here with chapter 4, in which I introduce hypoxia-ischemia as a model of cerebral ischemia in mice, and examine mortality and the modes of brain injury as a consequence of this ischemic model. Assessments of cerebral blood flow and other systemic parameters are presented in chapter 5. In chapter 6, I will present detailed data on the neurophysiology of hypoxia-ischemia, assessed primarily with the intracranial electroencephalogram (EEG). In chapter 7, I address the main topic of this thesis; that is, early-onset post-ischemic seizures and the impact of these seizures on subsequent outcome. This chapter will rely on behavioral assessments of these seizures. In chapters 8 and 9, I will present data regarding the role of the EEG in detecting and predicting these seizures. Additionally, I have delegated some experimental results and reference material to the appendices. The information found in the appendices augments the main body of results, but it is not critical to an understanding of the main arguments.

4 Results I: HI induces mortality and structural brain injury

Hypoxia-ischemia (HI) is a model of cerebral ischemia that has been extensively employed for experimental stroke studies in rodents (Vannucci et al. 2001; O'Donnell et al. 2002; Olson and McKeon 2004). Here, I will present data on post-HI mortality and brain injury under my experimental conditions. Assessments of brain injury were carried out utilizing gross histological assessments with cresyl violet (Nissl stain); in addition, more specific examinations of cellular degeneration utilizing TUNEL and FluoroJade were also carried out. The concepts of post-HI
mortality and the division of the modes of post-HI brain injury developed in this opening section will be a recurrent theme throughout this thesis and will serve as a foundation for the investigation of post-HI seizures.

### 4.1 HI episodes and resulting mortality

Adult male C57BL/6 mice were subjected to a hypoxic-ischemic (HI) episode consisting of a permanent ligation of the right common carotid artery (RCCA) followed by a global hypoxic episode (8% O₂, ~30 minutes). The hypoxic episode was conducted mainly in non-anesthetized animals (unless otherwise indicated) in an attempt to avoid the possible confounding effects of anesthetics on seizure genesis and brain injury.

Following ligation of the RCCA under isoflurane anesthesia, mice typically regained consciousness within 5 minutes. No obvious abnormal behavior was noted following RCCA ligation. The hypoxic episode was conducted 1.5 hours later (unless otherwise indicated) when mice had regained ‘normal’ behaviors such as grooming and exploration. During the hypoxic episode, the animals usually exhibited a brief period of arousal and movement, but were generally immobile throughout the hypoxic episode. The majority of animals tolerated the 30-minute hypoxic episode, but hypoxia was immediately terminated if animals showed evident respiratory depression or distress. Four animals died during the hypoxic episode, and were excluded from further analysis. All remaining animals were included in this study. After the hypoxic episode, the animals were monitored by a combination of visual inspection and video camera recording, and were allowed to survive for up to 6 weeks prior to histological assessments.

At last census, mortality had occurred in 30.9% (26 of 84) of mice within 72 hours following HI. Mortality was defined as either spontaneous death (15 mice) or mandatory euthanization (11 mice) in compliance with ethical guidelines (see Methods 3.2). The mice that died were typically immobile for at least 30 minutes following the hypoxic episode. Within 30 minutes to 2 hours following the hypoxic episode, these mice exhibited persistent rotation in short circles to the right; in some cases this persisted for hours, and was eventually followed by long periods of immobility. These circular rotations and/or bouts of immobility were frequently interrupted by convulsions (see Chapter 7). These animals were generally immobile for several hours prior to death or mandatory euthanization, and did not respond to touch or a lateral push, and they
exhibited a lack of grooming, exploration, feeding, and drinking. Animals exhibiting this type of behavior were euthanized under ethical guidelines. In mice exhibiting spontaneous mortality, the direct cause of death was not determined (these mice died during overnight recording sessions, and hence evaded mandatory euthanization under ethical guidelines). Mortality and physical impairment was not observed in sham operated animals (n=23) or in mice subjected to RCCA ligation alone (n=14).

Of the 11 mice euthanized within 72 hours post-HI, histological assessments of brain injury were carried out in 8 mice. Extensive injury to the hemisphere ipsilateral to the RCCA ligation, but not the contralateral hemisphere, was qualitatively observed in all 8 mice. Hereafter, the terms ‘ipsilateral’ and ‘contralateral’ are in reference to the RCCA ligation. Such ipsilateral injury manifested as weak cresyl violet staining throughout the ipsilateral hemisphere, which indicates extensive cell injury (Figure 3B). These results suggest that post-HI mortality and poor physical outcome are strongly associated with severe ipsilateral brain injury.

### 4.2 Histological assessments of brain injury at later post-HI times

Detailed histological assessments were conducted in 30 mice that survived for up to 6 weeks post-HI. Surviving mice did not display any evidence of obvious behavioral or motor deficits at this time-point. However, I confirmed that there is a sharp dichotomy in clear post-HI brain injury as previously described by others (Vannucci et al. 2001; O'Donnell et al. 2002; Olson and McKeon 2004; Kadam et al. 2010). In 36.7% of animals (11 of 30), no clear evidence of brain injury was observed in cresyl violet stained brain sections (Figure 3C). Tissue integrity was macroscopically similar to that observed in sham operated animals (n=5 mice; Figure 3A). In contrast, clear (visible to the naked eye) cystic infarcts were observed in the remaining 63.3% of animals (19 of 30). Infarcts were consistently observed throughout large regions of the ipsilateral hemisphere, including large portions of the cerebral cortex, hippocampus, striatum, and thalamus, while general tissue integrity was preserved in the contralateral hemisphere and cerebellum. In these animals, the infarcts consistently manifested as regions devoid of brain tissue; for instance, in more severe cases, the ipsilateral hippocampus was absent in the brain sections examined (Figure 3D).
Figure 3. Histological outcomes following hypoxia-ischemia

A. sham control

B. 24 hrs post HI

C. 6 weeks post HI

D. 6 weeks post HI
Figure 3. Histological outcomes following hypoxia-ischemia

A-D, coronal sections (8 µm thickness) were obtained from 4 mice, stained with cresyl violet and photographed under a dissecting microscope. Low power images on the left were taken at planes corresponding to Bregma -3.2 mm, -2.4 mm, -1.5 mm, -1.1 mm, -0.2 mm, 0.5 mm, 1.2 mm and 1.9 mm, (from left to right and top to bottom). Higher power images on the right were obtained from hippocampal regions at indicated coronal planes (filled circles). Ipsilateral and contralateral are relative to the ligation or sham isolation of the right common carotid artery. A, brain sections were obtained at 6 weeks post-sham operation. B, brain sections were obtained at 24 hours post-HI from an animal euthanized under ethical guidelines. Note the lighter staining in the ipsilateral hemisphere (right hemisphere) and diminished hippocampal cellular layers. C and D, sections were obtained at 6 weeks post HI. Note that gross brain structure is intact in C, whereas in D there is extensive tissue loss in the ipsilateral hemisphere (right hemisphere). Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
Figure 4. Quantification of post-HI brain injury
Figure 4. Quantification of post-HI brain injury

Injury of the ipsilateral hemisphere was quantified at the 8 coronal planes described above (n = 19 mice). Only animals exhibiting gross ipsilateral lesions as in Figure 1D were included for analysis. Infarcted regions were quantified as the % reduction in ipsilateral hemisphere area relative to contralateral hemisphere area. No significant differences in the extent of tissue loss was observed across the 8 coronal planes examined (p=0.443; One-Way ANOVA), suggesting a relatively uniform distribution of brain injury throughout the ipsilateral hemisphere. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
For the remainder of this thesis, ‘clear/extensive brain injury’ is defined as an ipsilateral hemisphere manifesting such large and obvious regions of tissue loss (i.e., panhemispheric injury), while ‘minimal/unclear brain injury’ is defined as a lack of clear brain injury in the ipsilateral hemisphere.

Quantification of infarct area was carried out only in animals that exhibited clear ipsilateral injury upon qualitative histological examination, and this revealed extensive loss of brain tissue in the ipsilateral hemisphere (relative to the contralateral hemisphere) when measured at 8 coronal planes (Figure 4). No significant differences in the extent of tissue loss were observed across the 8 coronal planes examined (p=0.443; One-Way ANOVA), suggesting a relatively uniform distribution of brain injury throughout the ipsilateral hemisphere.

Overall, the dichotomy in brain injury, incidence of infarction, extent of brain injury, and mortality rate following HI are in line with those reported by others (Vannucci et al. 2001; Olson and McKeon 2004; Adhami et al. 2006; Kadam et al. 2010).

4.3 TUNEL and FluroJade staining reveal subtle brain injury

The above observations indicate that a proportion of animals failed to demonstrate clear ipsilateral brain injury upon gross histological examination at 6 weeks post-HI. This sharp demarcation in outcome has similarly been reported by others employing this model of cerebral ischemia in rodents (Vannucci et al. 2001; Olson and McKeon 2004; Adhami et al. 2006; Kadam et al. 2010). However, gross histological examinations may fail to detect subtle brain injury, and this issue has not been fully explored within the context of HI in adult mice. Hence, more detailed assessments of brain injury were carried out, including TUNEL staining, a marker of apoptosis, and FluoroJade staining, a general marker for degenerating cells. These assessments were carried out at 72 hours post-HI, and TUNEL or FluoroJade stained sections were contrasted to cresyl violet stained sections from the same animal (Figures 5 and 6).

At 72 hours post-HI, mice did not exhibit the large cystic infarcts as described above; rather, the ipsilateral hemisphere was characterized by sparse and weak cresyl violet staining (Figure 5A, right). In animals that demonstrated this clear ipsilateral injury, a statistically significant increase in TUNEL (p≤0.022) and FluoroJade (p<0.001) positive signals was observed in the ipsilateral
temporal cortex relative to the contralateral cortex and sham controls (n=5 mice; One-way ANOVA; Student-Newman-Keuls test; Figures 6A and 6B).

The temporal cortex was chosen for quantification because this region is consistently injured following HI; however, extensive TUNEL and FluoroJade signals were also observed in other brain regions including the thalamus and throughout all regions of the hippocampus (data not shown). Thus, as previously reported in mice with clear post-HI ipsilateral injury (Olson and McKeon 2004), increased TUNEL and FluoroJade signals overlap the regions of brain injury demarcated by cresyl violet staining within 72 hours post-HI.

Interestingly, in 5 mice lacking clear ipsilateral brain injury (based on cresyl violet-stained sections; Figure 5A, middle), TUNEL and FluoroJade-positive signals were also observed (Figures 5B and 5C, middle). However, these signals were generally less extensive and less uniform in distribution compared to the animals with clear ipsilateral brain injury described above. When data from 5 mice were pooled together, there were significantly more FluoroJade-positive cells in the ipsilateral temporal cortex relative to the contralateral cortex and sham controls; however, the number of FluoroJade-positive cells was significantly lower relative to animals with clear ipsilateral lesions (p<0.001; n=5 mice; One-way ANOVA on ranks; Student-Newman-Keuls test; Figure 6B). Ipsilateral TUNEL signals were clearly evident in 2 of 5 mice examined; however, the pooled ipsilateral TUNEL signals were not significantly different from the contralateral hemisphere or sham controls (n=5 mice p≥0.144; One-Way ANOVA; Student-Newman-Keuls; Figure 6A).

These observations suggest that HI may result in a ‘spectrum’ of brain injuries post-HI, including a subtle mode of brain injury, characterized by sporadic and localized regions of cell death, which may evade detection by gross histological inspection with cresyl violet. A major caveat that should be noted is that due to the sporadic nature of the staining patterns in mice with minimal brain injury, it is likely the number of positive signals was overestimated due to bias in selecting imaging fields. Larger sample sizes and/or further markers are required to fully examine this issue. This was not a primary objective of this study. Nonetheless, the results quite clearly demonstrate that some animals exhibit a catastrophic panhemispheric infarction following HI, while other animals exhibit minimal/unclear brain injury post-HI.
Figure 5. TUNEL and FluoroJade assessments of post-HI injury

A  sham control  minimal injury  clear injury  
  cresyl violet

B  
  tunel

C  
  fluorojade

1 mm  100μm
Figure 5. TUNEL and FluoroJade assessments of post-HI injury

A, representative cresyl violet stained sections (20 µm thickness) from three mice obtained 72 hours post-sham or post-HI. *Left*, sham control; *Middle*, from a mouse that lacked clear ipsilateral brain injury post-HI (however, note some light staining in the ipsilateral cortex); *Right*, from a mouse demonstrating clear ipsilateral brain injury post-HI. Note the weak cresyl violet staining in the ipsilateral (right) hemisphere. *B* and *C*, representative TUNEL and FluoroJade stained sections from the ipsilateral temporal cortex grouped as in A. Images were acquired from the ipsilateral temporal cortex as approximately squared in A. Note the increased TUNEL and FluoroJade signals in animals that exhibited clear brain injury in cresyl violet sections. Moreover, in mice lacking clear brain injury in cresyl violet sections, TUNEL and FluoroJade signals were observed; however, these signals were generally less extensive and less uniform in distribution compared to the animals with gross ipsilateral brain injury described above. Hereafter, animals with the latter staining patterns are referred to as exhibiting ‘minimal/unclear’ ipsilateral brain injury. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
Figure 6. Quantification of TUNEL and FluoroJade signals in post-HI mice

A

TUNEL positive signals/mm²

sham  minimal injury  clear injury

B

fluorojade positive signals/mm²

sham  minimal injury  clear injury

contralateral  ipsilateral

*
Figure 6. Quantification of TUNEL and FluoroJade signals in post-HI mice

**A**, estimated number of TUNEL-positive signals in the ipsilateral temporal cortex of mice grouped as in Figure 3. In animals with clear ipsilateral brain injury evident in cresyl violet stained sections (n=5 mice), the number of ipsilateral TUNEL positive signals was significantly greater than that in the contralateral hemisphere, in sham controls (n=5 mice), and in post-HI mice with minimal/unclear brain injury (n=5 mice). *, p≤0.022; One-way ANOVA; Student-Newman-Keuls test. **B**, estimated number of FluoroJade-positive cells in the ipsilateral temporal cortex of mice grouped as in Figure 3. In animals with clear ipsilateral brain injury evident in cresyl violet stained sections, the number of ipsilateral FluoroJade positive signals was significantly greater than that in the contralateral hemisphere, in sham controls (n=5 mice), and in post-HI animals with minimal/unclear brain injury (n=5 mice). Furthermore, in contrast to TUNEL signals, a significant increase in ipsilateral FluoroJade signals was observed in mice with minimal/unclear ipsilateral brain injury relative to that in the contralateral hemisphere and in sham controls. However, these signals were generally less extensive and less uniform in distribution compared to the animals with clear ipsilateral brain injury described above. *, p<0.001; One-way ANOVA; Student-Newman-Keuls test. These observations suggest that HI may result in a ‘spectrum’ of brain injuries post-HI, including a subtle mode of brain injury, characterized by sporadic and localized regions of cell death, which may evade detection by gross histological inspection with cresyl violet. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
4.4 Summary of chapter 4

To sum up, there is a 30.9% mortality rate in post-HI mice. Moreover, based on histological assessments, post-HI mice were qualitatively divided by a blind experimenter into two categories. Approximately 71% of post-HI animals demonstrated clear and extensive brain injury (visible to the naked eye) throughout most of the ipsilateral hemisphere. Remaining post-HI animals did not demonstrate obvious injury in cresyl violet stained sections. However, in these animals, evidence of subtle cellular degeneration was observed in the ipsilateral hemisphere at 72 hours post-HI in FluoroJade stained sections; yet the extent of degeneration was significantly lower than in the former group of injured animals.

For the remainder of this thesis, I will employ the term ‘clear/extensive brain injury’ to describe animals that exhibited clear extensive ipsilateral panhemispheric brain injury post-HI, and I will employ the term ‘minimal/unclear brain injury’ to describe animals that did not demonstrate panhemispheric infarction post-HI. Although the latter group may exhibit subtle brain injury that may have been missed by gross histological examination with cresyl violet, this caveat does not affect the interpretation of later presented data and testability of later proposed hypotheses.
Chapter 5

5 Results II: Potential factors that influence post-HI outcome

The previous chapter has indicated that there is a dichotomy in brain injury following hypoxia-ischemia (HI). Some animals exhibit extensive panhemispheric ipsilateral brain injury that is clearly evident in cresyl violet stained sections, while other animals are seemingly spared of this outcome. This observation is in line with previous studies utilizing this model. However, this particular issue has not been effectively addressed. HI is a two-step procedure that involves occlusion of the right common carotid artery (RCCA) followed by respiratory hypoxia (8%O₂), and this hypoxic episode may have complex systemic effects that could potentially influence outcome.

Thus, the objectives of this section are 1) To confirm that cerebral blood flow is decreased during HI; thus replicating the results of Adhami et al (2006). 2) To explore whether the magnitude of cerebral ischemia during HI could account for the observed dichotomy in brain injury, which is an issue that has not been examined 3) To determine whether dramatic alterations in body temperature and cardiac function can explain the variability in post-HI brain injury.

5.1 Cerebral blood flow

Cerebral blood flow (CBF) was measured using a laser Doppler system in anesthetized mice. Local CBF was estimated by a Doppler probe through the exposed intact skull (see Methods 3.6), which likely measured local cortical blood flow in a field of 0.5mm in diameter. The technique is based on the Doppler-shift in wavelength of reflected light caused by moving blood cells, and this method is well-established in experimental and clinical practice. For these experiments, animals were subjected to HI under continuous isoflurane anesthesia, and body temperature was monitored by a rectal probe and maintained at ~37°C via a heating bag. Doppler measurements revealed no consistent change in CBF in sham operated mice (n=5). In contrast, the HI episode caused significant reductions of ipsilateral CBF (n=10 mice; Figure 7). These
mice were sacrificed 7-10 days later for histological assessments of ipsilateral brain injury. Of the 10 mice subjected to HI, 5 animals exhibited clear brain injury in the ipsilateral hemisphere as described above. When the CBF measurements were grouped based on subsequent histological outcomes, the overall decrease by combined RCCA ligation and hypoxia was greater in the animals with clear ipsilateral brain injury than those with minimal/unclear injury (to 10.7±1.2% vs. 35.4±6.8% of baseline, respectively; p=0.002; One-Way ANOVA; Student-Newman-Keuls test; Figure 7, right). The reductions of CBF by the RCCA ligation alone were not statistically different between the two groups (to 53.3±4.7% vs. 45.2±4.2% of baseline, respectively; p=0.238; One-Way ANOVA; Student-Newman-Keuls test). The latter negative result should be interpreted with caution, and larger sample sizes are required to fully address this issue.

Nonetheless, the results support previous studies that have demonstrated an approximately 50% decline in ipsilateral cortical blood flow as a consequence of unilateral occlusion of the common carotid artery in mice (Adhami et al. 2006; Todo et al. 2008; Yoshizaki et al. 2008). This decline has been demonstrated to be stable for up to 3 months following the occlusion (Yoshizaki et al. 2008). More importantly, in my experiments, superimposing respiratory hypoxia resulted in a further decline in CBF, thus reproducing the results of Adhami et al (2006). This indicates that clear panhemispheric post-HI brain injury is a consequence of severe cerebral ischemia. Finally, this is the first demonstration of variable reductions in CBF and the correlation to post-HI brain injury in adult mice. As a caveat, it should be noted that Doppler-based measurements indirectly examine CBF in the superficial cerebral cortex, while alterations in CBF in deeper brain regions such as the hippocampus would not be measured with this approach.
Figure 7. HI-induced changes in cerebral blood flow
Figure 7. HI-induced changes in cerebral blood flow

Laser Doppler measurements of cerebral blood flow in ipsilateral hemispheres. *Left*, representative measurements were collected from a sham operated mouse (top) and 2 mice (middle and bottom) following ligation of the right common carotid artery (RCCA, arrow) and hypoxic episodes (thick line). Measurements are presented as % of baseline. Note a more substantial decrease in ipsilateral cerebral blood flow during hypoxia in one mouse (black trace) relative to another mouse (gray trace). *Right*, measurements were quantified as % change relative to a 3-min baseline after the occlusion of the RCCA and at the end of hypoxia or 1-hour control monitoring. Data were grouped for mice with or without clear ipsilateral brain injury. *, p=0.002, One-Way ANOVA; Student-Newman-Keuls test. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
5.2 Body temperature

Alterations in body temperature have been described in experimental models of stroke (Williams et al. 2004; Williams et al. 2006), and potential alterations in body temperature may influence brain injury following cerebral ischemia. In my experiments, the chamber for the hypoxic challenge was warmed to keep inner air temperature at 35-35.5°C (Vannucci et al. 2001; Olson and McKeon 2004) in an attempt to prevent or minimize potential hypothermia that might occur during the hypoxic episode. However, there are no detailed studies of body temperature in non-anesthetized mice during HI.

To explore whether changes in body temperature may influence the post-HI outcomes described above, intraperitoneal body temperature was measured from non-anesthetized animals using a telemetry system (Figure 8). Animals were continuously monitored before and following the hypoxic episode, and allowed to recover for up to 7 days before histological assessments of brain injury. Data were grouped for animals with (n=7) and without (n=7) clear injury in ipsilateral hemisphere. A significant increase in body temperature was noted in animals with clear injury at the end of the hypoxic episode (38.7±0.4°C; p<0.001) and at 15 minutes post-hypoxia (38.6±0.4°C; p<0.001) relative to animals with minimal/unclear brain injury, but not at other time-points examined (p>0.144; Two-way repeated measures ANOVA; Student-Newman-Keuls test; Figure 8).

These observations suggest that increased body temperature during and shortly after the hypoxic episode may contribute to post-HI brain injury and the variability in post-HI brain injury. The moderate hyperthermia is in keeping with that described in rats subjected to middle cerebral artery occlusion and in humans following stroke (Regoldi et al. 2000a; Williams et al. 2004; Williams et al. 2006; Hallows et al. 2010) and may be due to damage or dysfunction in thermoregulatory centers of the hypothalamus (Regoldi et al. 2000a).
Figure 8. HI-induced changes in body temperature

![Graph showing body temperature changes during hypoxia and post-hypoxic period.](image)

Graph legend:
- **Grey** bars: minimal injury (n=7 mice)
- **Black** bars: clear injury (n=7 mice)
Figure 8. HI-induced changes in body temperature

Telemetry measurements of intra-peritoneal temperatures in non-anesthetized mice. *Left*, representative measurement from a mouse with later recognized clear ipsilateral brain injury. A continuous data section was collected before (post-RCCA occlusion) and following the hypoxic episode (thick line). *Right*, a summary of telemetric temperature measurements. Data were collected at 10 days post implantation (baseline) and at different times, i.e., at 1.5 hour post RCCA occlusion (o), at the end of hypoxia (h), 15 min post hypoxia (p) and 18–24 hours later. Data were grouped for mice with or without clear brain injury. *, p<0.001; Two-way repeated measures ANOVA; Student-Newman-Keuls test. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
Figure 9. Rectal Temperature during HI in anesthetized mice
Mice were subjected to HI under anesthesia (2% isoflurane). Rectal temperature was measured continuously during the hypoxic episode. The duration of hypoxia is indicated by a dashed line. During the HI episode, the animals were warmed by a heating pad to maintain their rectal temperatures at ~37°C. Mice were sacrificed 7-10 days later for histological assessments of ipsilateral brain injury. Rectal temperature is presented in 1 minute increments for mice with (black squares; n=5) and without (white squares; n=4) later recognized ipsilateral infarcts. No evidence of hyperthermia was observed; however, in animals that subsequently demonstrated clear ipsilateral brain injury, a transient, uncontrollable reduction in body temperature during HI was observed. This is in contrast to what was observed in non-anesthetized mice (see Figure 8). * p<0.05; One-Way repeated measures ANOVA; Student-Newman-Keuls test. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
However, a number of caveats should be noted before interpreting these results. Firstly, in animals subjected to HI under isoflurane anesthesia, hyperthermia was not observed during the course of HI; on the contrary, rectal temperature was transiently and significantly decreased by approximately 1°C in animals with clear ipsilateral brain injury relative to those with minimal/unclear brain injury (Figure 9). Secondly, measurements of intraperitoneal and rectal temperatures may not adequately represent brain temperature. Thirdly, sham-operated animals were not included in these experiments. (Sham operated animals were not included due to the difficulty and cost of telemetry recordings and because the interest was in group differences associated with brain injury.) Further studies are required to fully examine the effects of body temperature.

5.3 Heart rate

Heart rate has not been examined in non-anesthetized mice during HI. To explore whether hypoxia-induced alterations in heart rate may explain the variable outcome following HI, telemetry electrocardiogram (ECG) recordings were carried out to examine heart rate in non-anesthetized mice (Figure 10). The changes in heart rate were similar in animals with (n=4) and without (n=2) clear injury in the ipsilateral hemisphere; therefore, data were pooled together. Heart rate was significantly increased from baseline when measured at the end of hypoxia (from 475.5±63.9 to 712.6±29.4 beats per min, p<0.001, One-Way repeated measures ANOVA; Student-Newman-Keuls test; Figure 10, below).

This increase might reflect a transient compensatory mechanism to boost cardiac output under hypoxic stress, as heart rate quickly returned to baseline levels when measured at 10 min after termination of the hypoxic episode. Alternatively, the increase in heart rate may be associated with a stress or a fear-related response as these animals were non-anesthetized. However, as sham-operated animals were not included in this experiment, the above two propositions cannot be distinguished. (Sham operated animals were not included due to the difficulty and cost of

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6 The cardiovascular physiology of the mouse has been well-studied (Hoyt et al. 2007). Resting heart rate of the conscious mouse lies between 450 and 550 beats/min. On a body weight basis, stroke volume is around 1 µl/g, which is similar to rats (~250 µl in 250-g animals) and humans (~60 ml in a 70 kg person). To put the changes in heart rate observed during hypoxia into context, it should be noted heart rates as high as 800 beats/min can occur even during routine activities such as grooming, eating, and handling. Furthermore, there also appears to be low
telemetry recordings and because the interest was in group differences associated with brain injury.) Taken together, the results suggest that alterations in cardiac function cannot fully explain the dichotomy in outcome following HI. However, further studies with more detailed assessments of cardiac function such as cardiac output, the presence of arrhythmias, detailed analysis of ECG waveforms, measurements of blood pressure, and analyses of arterial and venous blood gases are required to fully rule out the possibility of cardiovascular dysfunction.

tagal parasympathetic tone in control of the resting mouse heart. This suggests that autonomic control of the resting mouse heart rate is predominately sympathetic, which is different from what occurs in humans.
Figure 10. HI-induced changes in heart rate

ECG

baseline

0.5 mV

0.5 sec

heart beats (100/min)

n=6
Figure 10. HI-induced changes in heart rate

Telemetric electrocardiogram (ECG) in non-anesthetized mice. The changes in heart rate were similar in animals with (n=4) and without (n=2) clear injury in the ipsilateral hemisphere; therefore, data were pooled together. Above, representative measurement from a mouse with later recognized clear ipsilateral brain injury. Below, heart rates were measured at 10 days post implantation (baseline) and at different times, i.e., at 1.5 hour post RCCA occlusion (o), at the end of hypoxia (h), 15 min post hypoxia (p) and 18-24 hours later. Data are grouped for mice with or without injury. *, p<0.001; One-way repeated measures ANOVA; Student-Newman-Keuls test. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
5.4 Summary of chapter 5

In summary, the CBF measurements are in accordance with a previous study (Adhami et al. 2006) demonstrating that HI results in a significant decline in ipsilateral CBF. However, in addition to the work of Adhami et al. (2006), the above results suggest that variability in cerebral blood flow may explain the dichotomy in post-HI brain injury. In addition, body temperature and heart rate were assessed in non-anesthetized mice subjected to HI. The data indicates that there are transient changes in both these parameters. However, these systemic alterations are unlikely to be major determinants of the ipsilateral brain injury described above. Although more detailed studies are required to fully examine the systemic alterations that arise following HI, I suggest that differences in the extent of cerebral ischemia are largely responsible for the two distinct histological outcomes presented in chapter 4.
Chapter 6

6 Results III: HI results in EEG suppression and synaptic dysfunction

The previous chapters have indicated that there is a dichotomy in brain injury following hypoxia-ischemia (HI). Some mice exhibited catastrophic panhemispheric ipsilateral brain injury that is clearly evident in cresyl violet stained sections, while other animals were seemingly spared of this outcome. Furthermore, the magnitude of the reduction in cerebral blood flow was found to be a predictive parameter for subsequent brain injury. This indicates that HI results in variable cerebral ischemia. However, Doppler-based measurements of cerebral blood flow are limited for three main reasons: 1) The recordings must be carried out under anesthesia, which may influence HI brain injury. 2) The recordings reflect local cortical blood flow, while deeper brain regions are not examined with this approach. 3) It is difficult, if not practically impossible, to carry out long-term chronic assessments of cerebral blood flow. These issues may be circumvented by intracranial EEG recordings in free-moving, non-anesthetized animals.

Here, I present data examining the effects of HI on intracranial EEG recordings from free-moving, non-anesthetized adult C57BL/6 mice. The EEG is believed to primarily reflect local synaptic potentials in the brain, and is therefore a potential marker for continual in vivo assessments of ongoing synaptic activity. The rationale behind the employment of EEG recordings is three-fold: 1) To my knowledge, an examination of EEG dysfunction has not been carried out in the context of HI in adult mice; moreover, detailed, long-term experimental investigations of EEG activity during and following experimental cerebral ischemia are, in my opinion, generally lacking. 2) EEG recordings have demonstrated potential utility in assessing outcome following cerebral ischemia in animals and in humans; however, the utility of quantitative EEG remains contentious. 3) As the EEG is commonly employed in the detection and study of seizures, an examination of the effects of HI on basic EEG parameters seems prudent as a prelude to examining EEG activity during post-ischemic seizures.
6.1 EEG characteristics in free-moving mice

Bilateral intracranial EEG recordings from non-anesthetized, free-moving mice were conducted as described previously (Wu et al. 2008; Wais et al. 2009; D’Cruz et al. 2010). Intracranial EEG recordings are superior to scalp or sub-dural recordings as they permit an investigation of deep brain structures. The majority of EEG recordings were carried out simultaneously from the right hippocampal CA1 region and left somatosensory cortex, with a reference electrode in the ipsilateral motor cortex (Figure 11; see Methods 3.4.1). This configuration of recording electrodes was chosen as it has permitted a reliable detection of EEG ictal discharges in juvenile mice following hypoxic challenges (Wais et al. 2009) and in adult rats following intra-hippocampal injections of cobalt (He et al. 2009). Additionally, bilateral hippocampal and bilateral cortical recordings were also carried out.

Importantly, brain region-specific, behavioral state-dependent EEG signals were consistently observed in adult C57BL/6 mice as previously described in rodents (Leung 1992; Leung 1998; Buzsáki et al. 2003; Leung and Shen 2004; Buzsáki 2006; O’Keefe 2007; Wu et al. 2008; Wais et al. 2009; D’Cruz et al. 2010; Figures 11B and 11C). For example, when mice were immobile/quiescent, the hippocampal EEG was dominated by ‘large-irregular activity’, including intermittent sharp waves, whereas the cortical EEG was dominated by slow waveforms in the delta frequency band (1-4 Hz). Dominant hippocampal and cortical frequencies during immobile/quiescent periods were 1.95±0.15Hz and 2.01±0.16Hz, respectively; corresponding peak power was 0.025±0.01 mV²/Hz and 0.024±0.01mV²/Hz, respectively (n=20 mice). In contrast, when mice were moving or exhibiting exploratory behavior, the hippocampal EEG manifested a robust rhythmic activity in the theta range (4-12 Hz, theta rhythm), whereas the cortical EEG displayed low amplitude, desynchronized activity (Figures 11B and 11C). Dominant hippocampal frequency during mobility/exploration was 7.90±0.12Hz and peak power was 0.016±0.01 mV²/Hz (n=29 mice).

EEG waveforms are therefore complex signals, which vary in frequency and amplitude in correlation with ‘behavioral states’. The EEG rhythms and dynamics I have observed are consistent with previous studies in rodents; thus validating the EEG recording method utilized. However, the complexity of the EEG signal poses challenges in its potential application to quantitative assessments of ischemic brain injury. This issue is addressed next.
Figure 11. Brain region-specific and behavioral state-dependent EEG signals in adult mice

A
left cortex
right hippocampus

B
immobility
movement

hipp
cctx

C
immobility
movement

mv^2/Hz

frequency (Hz)
Figure 11. Brain region-specific and behavioral state-dependent EEG signals in adult mice. 

A, Representative brain sections (stained with cresyl violet) were obtained from a mouse with implanted electrodes in the left cortex and right hippocampus. EEG electrode tracks are indicated by arrows. B, EEG recording from a mouse with electrodes in the left cortex and right hippocampus. The trace illustrates continuous EEG activity during a period of immobility (left) and a period of mobility (right). When the mouse was immobile, the hippocampal EEG was dominated by so-called “large-irregular activity”, including intermittent sharp waves, whereas the cortical EEG was dominated by slow waveforms in the delta band (1-4 Hz, delta waves). In contrast, when the mouse was moving, the hippocampal EEG manifested a robust rhythmic activity in the theta range (4-12 Hz, theta rhythm), whereas the cortical EEG displayed low amplitude, desynchronized activity. C, power spectral plots for the hippocampus and cortex (black and gray lines respectively) during immobility (left) and movement (right) generated from the traces in B. Figure modified from EL-Hayek et al (2011b) with permission from Elsevier.
6.2 Ipsilateral EEG suppression in mice subjected to HI

The use of quantitative EEG in examining outcome following cerebral ischemia is not standardized in clinical or experimental practice. To my knowledge, there is no universally accepted parameter for investigating EEG alterations following cerebral ischemia. Moreover, in my opinion, no single quantitative variable can fully describe the complexity of the EEG signal, its multiple oscillatory components, its amplitude, and brain state-varying dynamics.

However, given the close physiological coupling of synaptic activity to cerebral blood flow, the suppression of EEG activity is believed to reflect the extent of cerebral ischemia in animal models (Raffin et al. 1991; Lu et al. 2001; Hartings et al. 2003) and humans (Jordan 2004; Firedman et al. 2009; Friedman et al. 2010), and EEG amplitude is employed clinically as a parameter to assess outcome following hypoxic-ischemic encephalopathy in neonatal humans (Shalak et al. 2003; Tao and Mathur 2010). I thus conducted intracranial EEG recordings in free-moving, non-anesthetized mice; and I recorded bilateral hippocampal and/or cortical EEG signals; measured the extent of EEG suppression for up to 6 weeks post-HI, and then correlated these measurements to brain injury and mortality.

There is currently no universally accepted parameter for investigating EEG suppression. Therefore, to quantify EEG suppression, I investigated four aspects of the mouse intracranial EEG. I have divided these four aspects under two headings below. Firstly, I will present 'amplitude-based' measurements of EEG amplitude and variance. Secondly, I will present 'Fast Fourier Transform (FFT)-based' measurements of EEG activity utilizing the root mean square (RMS) of the EEG power spectrum and investigations of hippocampal theta rhythms. EEG amplitude, variance, and RMS were carried out during periods of immobility; while examinations of theta rhythms were carried out during periods of navigation/exploration (see Methods 3.4.2).
Figure 12. EEG signals in a mouse with clear ipsilateral brain injury post-HI
Figure 12. EEG signals in a mouse with clear ipsilateral brain injury post-HI

Intracranial electroencephalogram (EEG) recordings in a non-anesthetized mouse that exhibited clear ipsilateral brain injury post-HI. Recordings were performed simultaneously from the ipsilateral hippocampus and contralateral cortex. Data segments were collected at 7 days post-implantation (baseline), following right common carotid artery (RCCA) ligation, during the entire course of the hypoxic episode, and at different times post-HI. Lower traces are time-expanded segments. Note that the ipsilateral hippocampal EEG signal is extremely suppressed during the hypoxic episode and remains suppressed throughout the recording period up to 6 weeks post-HI. Furthermore, note that contralateral cortical EEG activity is not appreciably affected.
Figure 13. EEG signals in a mouse with minimal ipsilateral brain injury post-HI
Figure 13. EEG signals in a mouse with minimal/unclear ipsilateral brain injury post-HI

Intracranial electroencephalogram (EEG) recordings in a non-anesthetized mouse with minimal/unclear ipsilateral brain injury post-HI. Recordings were performed simultaneously from the ipsilateral hippocampus and contralateral cortex. Data segments were collected 7 days post-implantation (baseline), following right common carotid artery (RCCA) ligation, during the entire course of the hypoxic episode, and at different times post-HI. Lower traces are time-expanded segments. Note that the ipsilateral hippocampal EEG signal is mildly suppressed during the hypoxic episode but recovers to baseline levels throughout the recording period up to 6 weeks post-HI.
Figure 14. EEG signals in a sham-control mouse
**Figure 14. EEG signals in a sham-control mouse**

Intracranial electroencephalogram (EEG) recordings in a non-anesthetized sham control mouse. This mouse received right common carotid artery (RCCA) isolation but not ligation, and was subjected to air flow instead of hypoxia. Recordings were performed simultaneously from the ipsilateral hippocampus and contralateral cortex. Data segments were collected 7 days post-implantation (baseline), following sham surgery, during the entire course of the sham air flow, and at different times post-sham HI. Lower traces are time-expanded segments. Large amplitude events in upper traces are movement artifacts. Note that EEG activity was not appreciably affected by sham-operation throughout the recording period up to 6 weeks post-HI.
6.2.1 Amplitude-based measurements of EEG suppression

The objective here was to determine whether the magnitude of EEG suppression varies as a function of brain injury/mortality, and whether EEG amplitude and variance can serve as valid markers for EEG activity during and following HI in adult mice. Mice were divided into three groups: sham control mice, mice with clear brain injury/mortality post-HI, and mice with ‘minimal/unclear’ brain injury post-HI.

HI resulted in a marked suppression of EEG amplitude, and the magnitude and duration of EEG suppression was related to post-HI outcome (Figures 12-14). Specifically, in animals with clear ipsilateral brain injury or acute mortality (n=17 mice at baseline; Figure 12), the ipsilateral hippocampal mean EEG amplitude was significantly decreased at the end of the hypoxic episode (to 28.5±4.1% of baseline) and remained significantly decreased at 1 hour, 1 week, and 4-6 weeks later relative to both sham control mice (p<0.001) and to mice with minimal/unclear brain injury post-HI (p<0.001; Two-way ANOVA; Student-Newman-Keuls test; Figure 15). Similarly, the ipsilateral hippocampal EEG variance was significantly decreased at the end of the hypoxic episode (to 8.5±2.2% of baseline) and remained significantly decreased at 1 hour, 1 week, and 4-6 weeks later relative to both sham control mice (p<0.001) and to mice with minimal/unclear brain injury post-HI (p<0.001; Two-way ANOVA; Student-Newman-Keuls test; Figure 16). The above observations indicate that a profound and protracted suppression of ipsilateral EEG activity is associated with clear ipsilateral brain injury and mortality.

In contrast, in animals with minimal/unclear brain injury post-HI (n=14 mice at baseline; Figure 13), the mean amplitude of ipsilateral hippocampal EEG signals was significantly decreased at the end of the hypoxic episode relative to sham control mice (to 60.9±9.8% of baseline; p=0.013) but recovered by 1 hour post-HI (p=0.116) and at later time-points examined (p≥0.1118; Two-way ANOVA; Student-Newman-Keuls test test; Figure 15). Likewise, the mean variance of ipsilateral hippocampal EEG signals was significantly decreased at the end of the hypoxic episode relative to sham control mice (to 48.8±9.8% of baseline; p=0.004) but recovered by 1 hour post-HI (p=0.052) and at later time-points examined (p≥0.247; Two-way ANOVA; Student-Newman-Keuls test; Figure 16). These observations indicate that a relatively mild, transient, and reversible suppression of ipsilateral EEG activity is associated with minimal/unclear ipsilateral brain injury and survival.
Figure 15. Ipsilateral EEG amplitude as a measure of HI-induced EEG suppression
Figure 15. Ipsilateral EEG amplitude as a measure of HI-induced EEG suppression

Ipsilateral hippocampal EEG amplitude was calculated from sham control mice, mice with minimal/unclear ipsilateral brain injury post-HI, and mice with clear ipsilateral brain injury/mortality post-HI. Data is expressed as percentage of baseline and statistically significant group differences are indicated. Note that in animals with later recognized clear ipsilateral brain injury or mortality, EEG amplitude was strongly reduced during HI, and remained reduced throughout the recording period up to 6 weeks post-HI. In contrast, in animals lacking clear brain injury post-HI, EEG activity was mildly and reversibly suppressed. Due to mortality and insufficient data, some time-points had lower sample sizes than others. *, p≤0.013; Two-way ANOVA; Student-Newman-Keuls test. Figure modified from EL-Hayek et al (2011b) with permission from Elsevier.
Figure 16. Ipsilateral EEG variance as a measure of HI-induced EEG suppression
Figure 16. Ipsilateral EEG variance as a measure of HI-induced EEG suppression

Ipsilateral hippocampal EEG variance was calculated from sham control mice, mice with minimal/unclear ipsilateral brain injury post-HI, and mice with clear ipsilateral brain injury/mortality post-HI. Data is expressed as percentage of baseline and statistically significant group differences are indicated. Note that in animals with later recognized ipsilateral brain injury or mortality, EEG variance was strongly reduced and remained reduced throughout the recording period up to 6 weeks post-HI. In contrast, in animals with minimal/unclear brain injury post-HI, EEG activity was mildly and reversibly suppressed. Due to mortality and insufficient data, some time-points had lower sample sizes than others. *, p≤0.004; Two-way ANOVA; Student-Newman-Keuls test. Figure modified from EL-Hayek et al (2011b) with permission from Elsevier.
I next examined contralateral cortical EEG activity utilizing an identical strategy.

No significant group differences in the percent change of either contralateral EEG amplitude or variance was observed between sham control mice (n=7), mice with clear brain injury/mortality post-HI (n=10 mice at baseline), and mice with minimal/unclear injury post-HI (n=10 mice at baseline) at any of the time-points examined (p≥0.113 for amplitude and p≥0.073 for variance; Two-way ANOVA; Student-Newman-Keuls test; Figures 17 and 18). These observations indicate a lack of clear suppression in contralateral EEG activity, and supports previous observations of an absence of detectable contralateral brain injury.

However, I concede that subtle alterations in contralateral EEG activity may not have been detected due to the relatively small sample size and due to the generality of the parameters chosen for quantification. Moreover, the primary method of choice for presenting and analyzing data in this thesis is to examine percent changes relative to baseline and to then examine group differences; for the most part I have avoided comparisons of raw data within groups, as these differences generally mirrored differences across groups (see Methods 3.9 for justifications). This is not the case here. In this case, contralateral EEG amplitude and variance were significantly reduced relative to baseline at 1 hour and 1 day post-HI in animals with clear ipsilateral brain injury (p≤0.025; Two-way ANOVA; Student-Newmans-Keuls test; data not illustrated). Furthermore, as a caveat, it should be noted that drawing direct parallels between ipsilateral hippocampal activity and contralateral cortical activity is not entirely justifiable, as the hippocampus and cerebral cortex are different brain regions. However, as histological assessments, including cresyl violet, TUNEL and FluoroJade, and in vitro electrophysiological assessments (see 6.3 below) have clearly demonstrated a lack of contralateral brain injury, I abandoned further examinations of contralateral EEG activity: the effect of HI on contralateral EEG activity is not a primary goal of this study, nor does it alter the interpretation of the primary hypotheses.

Collectively, these observations clearly indicate that a relatively severe and prolonged suppression of ipsilateral EEG amplitude and variance are associated with clear ipsilateral brain injury and mortality, while a relatively milder and transient suppression of ipsilateral EEG activity during HI is associated with survival and a lack of extensive brain injury.
Figure 17. Contralateral EEG amplitude is minimally altered by HI

Contralateral EEG amplitude (% of baseline)

- end of HI
- 1 hour
- 1 day
- 1 week
- 4-6 weeks

- sham control (n=7 mice)
- minimal/unclear injury (n=6-10 mice)
- injury/mortality (n=4-10 mice)
Figure 17. Contralateral EEG amplitude is minimally altered by HI

Contralateral cortical EEG amplitude was calculated from sham control mice, mice with minimal/unclear brain injury post-HI, and mice with clear brain injury/mortality post-HI. Data is expressed as percentage of baseline. No statistically significant group differences were noted (p≥0.113; Two-way ANOVA; Student-Newman-Keuls test). Due to mortality and insufficient data, some time-points had lower sample sizes than others.
Figure 18. Contralateral EEG variance is minimally altered by HI
Figure 18. Contralateral EEG variance is minimally altered by HI

Contralateral cortical EEG variance was calculated from sham control mice, mice without brain injury post-HI, and mice with brain injury/mortality post-HI. Data is expressed as percentage of baseline. No statistically significant group differences were noted (p≥0.073; Two-way ANOVA; Student-Newman-Keuls test). Due to mortality and insufficient data, some time-points had lower sample sizes than others.
6.2.2 FFT-based measurements of EEG suppression

Mean amplitude and variance were sampled from a digital EEG signal in the time domain. However, these measurements preclude an analysis of the frequency components of the EEG. To incorporate an analysis of EEG activity in the frequency domain, I analyzed two parameters that I believe strongly supplement the above measurements of EEG amplitude and variance. The first measurement is the root mean square (RMS) of the EEG power spectrum. The RMS can be thought of as mean amplitude (in mV) in the frequency domain. A second parameter chosen for analysis is the hippocampal theta rhythm.

As described above, the same mice were divided into three groups: sham control mice, mice with clear ipsilateral brain injury/mortality post-HI, and mice with minimal/unclear ipsilateral brain injury post-HI. HI resulted in a suppression of EEG activity, which manifested as a reduction of the EEG RMS, and the magnitude and duration of EEG suppression was related to post-HI outcome. Specifically, in animals with clear ipsilateral brain injury or acute mortality (n=17 mice at baseline), the ipsilateral hippocampal EEG RMS was significantly decreased at the end of the hypoxic episode (to 22.5±5.4% of baseline) and remained significantly decreased at 1 hour, 1 week, and 4-6 weeks later relative to both sham control mice (p<0.001) and to mice with minimal/unclear brain injury post-HI (p≤0.004; Two-way ANOVA; Student-Newman-Keuls test; Figure 19).

In contrast, in animals with minimal/unclear brain injury (n=14 mice at baseline), the mean RMS of ipsilateral hippocampal EEG signals was significantly decreased at the end of hypoxic episode (to 55.4±10.8% of baseline; p=0.005) and at 1 hour post-HI (p=0.031) relative to sham control mice, but not at later time-points examined (p≥0.260; Two-way ANOVA; Student-Newman-Keuls test; Figure 19). The significance at 1 hour post-HI is in contrast to that observed with amplitude and variance presented above, suggesting that RMS is a more sensitive parameter for investigations of the suppression of EEG activity.

These observations support the previous measurements of EEG amplitude and variance, and indicate that a relatively severe and prolonged suppression of EEG RMS is associated with clear ipsilateral brain injury/mortality, while a relatively milder and transient suppression of EEG RMS during HI is associated with survival and a minimal/unclear brain injury.
Figure 19. Ipsilateral EEG RMS as a measure of HI-induced EEG suppression
Figure 19. Ipsilateral EEG RMS as a measure of HI-induced EEG suppression

Ipsilateral hippocampal EEG root mean square (RMS) was calculated from sham control mice, mice with minimal/unclear ipsilateral brain injury post-HI, and mice with clear ipsilateral brain injury/mortality post-HI. Data is expressed as percentage of baseline and statistically significant group differences are indicated. Note that in animals with later recognized clear ipsilateral brain injury or mortality, EEG RMS was significantly decreased at the end of the HI episode and remained decreased throughout the recording period. Further, in mice with minimal/unclear brain injury post-HI, EEG RMS was mildly and reversible reduced. Due to mortality and insufficient data, some time points had lower sample sizes than others. *, p≤0.032; Two-way ANOVA; Holm-Sidak test. Figure modified from EL-Hayek et al (2011b) with permission from Elsevier.
To further characterize the effects of HI on ipsilateral hippocampal EEG activity, I carried out an FFT-based analysis of the hippocampal theta rhythm, which is strongly implicated in spatial navigation and spatial memory functions of the rodent hippocampus (Buzsáki 2006; O'Keefe 2007; Figures 20 and 21). As above, animals were grouped into sham controls (n=7 mice), mice with clear ipsilateral brain injury/mortality post-HI (n=9 mice at baseline), and mice with minimal/unclear brain injury post-HI (n=13 mice at baseline).

In mice with clear ipsilateral brain injury/mortality post-HI, a significant reduction in theta frequency (to 5.73±0.36Hz) was observed at 1 day post-HI, and remained significantly decreased at 1 week and 4-6 weeks post-HI relative to sham control animals (p≤0.003) and to mice with minimal brain injury post-HI (p≤0.036; Two-way ANOVA; Student-Newman-Keuls test; Figure 21A). In mice with minimal brain injury post-HI, a significant reduction in theta frequency (to 6.89±0.16Hz) was observed at 1 day post-HI relative to sham controls (p=0.008; Two-way ANOVA; Student-Newman-Keuls test; Figure 21A) but not at later times examined (p≥0.087).

In animals that exhibited clear ipsilateral brain injury/mortality post-HI, a significant reduction of theta power (to 11.69±7.39% of baseline) was observed at 1 day post-HI, and remained significantly decreased at 1 week and 4-6 weeks post-HI relative to sham control animals (p≤0.006) and mice with minimal/unclear brain injury post HI (p≤0.001; Two-way ANOVA; Student-Newman-Keuls test; Figure 21B). Interestingly, in animals with minimal/unclear injury post-HI, a significant increase in theta power (to 150.60±25.74% of baseline) was observed at 1 day post-HI (p=0.037), but no significant alterations were observed at later times (p≥0.375; Two-way ANOVA; Student-Newman-Keuls test; Figure 21B).

Collectively, the above observations indicate that a prolonged reduction in theta frequency and power is associated with clear brain injury/mortality post-HI, while a transient and reversible reduction in theta frequency at 1 day post-HI coupled with an increase in power is associated with survival and minimal/unclear brain injury. These results augment the above-mentioned alterations in EEG amplitude, variance, and RMS, and they further support the utility of intracranial EEG recordings in assessing or predicting outcome following cerebral ischemia.
Figure 20. Disruption of hippocampal EEG theta rhythms in post-HI mice

A

baseline
4 wk post sham operation

post operation

B

baseline
6 wk post HI

mV²/Hz

0.012

0.008

0.004

0.000

0

10

20

frequency (Hz)

0.5 mV

1 sec

C

baseline
6 wk post HI

post HI

baseline
Figure 20. Disruption of hippocampal EEG theta rhythms in post-HI mice

A-C, EEG traces were collected from mice implanted bilaterally in the ipsilateral hippocampus and contralateral cortex. Representative EEG signals were recorded during periods of mobility. During periods of mobility, the hippocampal EEG is dominated by a theta rhythm (4-12Hz). Theta rhythms are implicated in spatial navigation functions of the rodent hippocampus. A, EEG activity in a mouse before and 4 weeks post sham operation. Corresponding hippocampal power spectra are shown at the far right. B, EEG theta activity in a mouse with clear ipsilateral brain injury post-HI. Note that ipsilateral rhythms are strongly attenuated post-HI. C, EEG theta activity in a mouse with minimal/unclear ipsilateral brain injury post-HI. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
Figure 21. Quantification of hippocampal theta rhythms in post-HI mice

A

 theta frequency (Hz)

<table>
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<th>4-6 wk later</th>
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<td>□</td>
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<tr>
<td>minimal/unclear injury</td>
<td>□ (n=9-13)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>injury</td>
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<td>□</td>
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</table>

B

 theta power (% of baseline)

<table>
<thead>
<tr>
<th></th>
<th>1 day later</th>
<th>1-2 wk later</th>
<th>4-6 wk later</th>
</tr>
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<tbody>
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<td>sham control</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>minimal/unclear injury</td>
<td>150*</td>
<td>150*</td>
<td>150*</td>
</tr>
<tr>
<td>injury</td>
<td>200*</td>
<td>200*</td>
<td>200*</td>
</tr>
</tbody>
</table>
Figure 21. Quantification of hippocampal theta rhythms in post-HI mice

A and B, respective quantifications of dominant theta frequency and power in sham control mice and in post-HI mice with or without clear ipsilateral brain injury. Power is expressed as percent of baseline. Statistically significant group differences are indicated. Note that both theta frequency and power are irreversibly reduced in animals that exhibited clear brain injury post-HI. Furthermore, in animals with minimal/unclear post-HI brain injury, theta frequency is transiently reduced while theta power is transiently increased at 1 day post-HI. Due to mortality and insufficient data, some time points had lower sample sizes than others.* p≤0.037; Two-way ANOVA; Student-Newman-Keuls test. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
6.3 Electrophysiological assessments in brain slices in vitro

To corroborate the above EEG and histological findings, in vitro electrophysiological recordings from acutely prepared brain slices were carried out. Recordings were carried out from the hippocampal CA1 region because the ipsilateral hippocampus is consistently injured following HI. CA1 field excitatory post synaptic potentials (EPSPs) were evoked by electrical stimulation of the Schaffer collateral pathway at near maximal intensity, and were recorded from slices that were prepared from both ipsilateral and contralateral hemispheres. Some slices were histologically processed to confirm hippocampal injury. Measurements from multiple slices were averaged for each animal, and data were grouped for animals with or without clearly recognizable hippocampal injury (Figure 22).

For the animals with clear hippocampal injury recognized either within 72 hours (n=3 mice that required mandatory euthanization) or 6 weeks (n=4 mice) post-HI, the amplitude of ipsilateral CA1 field EPSPs was severely depressed (Figure 22A). Because only a small number of mice were examined, data for both time-points were pooled together. Ipsilateral EPSP amplitude (0.15±0.05 mV; n=7 animals) was significantly smaller than both the contralateral (1.0±0.07 mV; p=0.002; One-way ANOVA; Student-Newman-Keuls test; n=13 animals) and sham control responses (1.27±0.08mV; p<0.001; One-way ANOVA; Student-Newman-Keuls test; n=8 animals; Figure 22B). In contrast, in the animals with minimal/unclear brain injury post-HI, the amplitudes of ipsilateral and contralateral CA1 field EPSPs were not significantly different from each other (0.93±0.12mV vs. 0.97±0.09 mV; p=0.997; One-way ANOVA; Student-Newman-Keuls test; n=7 animals) or from the sham controls (1.27±0.08mV, p=0.238; One-way ANOVA; Student-Newman-Keuls test; n=8 animals; Figure 22B).

These observations provide additional evidence indicating that HI induces clear ipsilateral brain injury in a proportion of adult mice under my experimental conditions.
Figure 22. *In vitro* electrophysiological assessments of HI brain injury

**A**

<table>
<thead>
<tr>
<th>Condition</th>
<th>CA1 f-EPSPs</th>
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<tbody>
<tr>
<td>sham</td>
<td></td>
</tr>
<tr>
<td>clear injury</td>
<td></td>
</tr>
<tr>
<td>minimal/unclear injury</td>
<td></td>
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</table>

contralat. | ipsilat.  
contralat. | ipsilat.

**B**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Peak amplitudes of f-EPSPs (mV)</th>
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<tbody>
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<tr>
<td>minimal/unclear injury (7)</td>
<td></td>
</tr>
<tr>
<td>clear injury (13)</td>
<td></td>
</tr>
</tbody>
</table>

contralat. | ipsilat.  
contralat. | ipsilat.

* (7)
Figure 22. *In vitro* electrophysiological assessments of HI brain injury

*A and B*, measurements of hippocampal CA1 field EPSPs in brain slices. *A*, representative responses were collected from 3 mice at 5-6 weeks post-sham surgery or HI. Each trace was averaged from 4-5 consecutive responses. *B*, the amplitudes of CA1 field EPSPs were measured from 3-4 slices per mouse, and data are presented for grouped mice. Numbers of mice examined are indicated. For animals with ipsilateral brain injury, preparation of ipsilateral hippocampal slices was possible in only 7 of 13 mice used (due to the extensive injury to the hippocampus). *, One-way ANOVA; Holm Sidak test; p≤0.002. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
6.4 Summary of chapter 6

The main points of this section can be summarized as follows. Firstly, adult C57BL/6 mice exhibited characteristic brain-region specific, behavioral state-dependent intracranial EEG signals. Secondly, HI resulted in a suppression of ipsilateral EEG activity and a disruption of hippocampal theta rhythms. Thirdly, both the magnitude of ipsilateral EEG suppression during HI and its recovery post-HI are good indicators of subsequent brain injury/mortality. Fourthly, there is general agreement across all the parameters chosen to quantify EEG suppression. Finally, in vitro electrophysiological assessments of synaptic transmission demonstrated impaired synaptic activity in post-HI animals; thus supporting the in vivo suppression of EEG activity and histological assessments of ipsilateral brain injury.
Chapter 7

7 Results IV: Early post-HI seizures are associated with mortality and brain injury

Here, I will present data describing the novel observation of early post-HI convulsive seizures and their association with mortality and brain injury. The effects of treatments with anticonvulsant drugs will also be presented. In this section, motor seizures are described solely according to behavioral assessments; I will defer a discussion of EEG activity during these seizures until chapter 8. I will also adopt the same terminology in regards to the modes of post-HI brain injury as described in previous chapters; namely, post-HI animals are divided into two groups: those with clear ipsilateral brain injury and those with minimal/unclear ipsilateral brain injury.

7.1 Characterization of post-HI motor seizures

Post-HI seizures were detected through a combination of video monitoring and visual inspection (see Methods 3.3). Of 76 animals examined, 33 mice (43.4%) exhibited at least two severe motor seizures within 72 hours post-HI. These convulsive seizures were characterized by generalized tonic-clonic convulsions, as well as by jumping, fast running, barrel rolling and/or falling with loss of the righting reflex. These seizures occurred while the animals were in a waking, immobile state, and they manifested with a sudden onset without noticeable preceding behavioral signs. In general, the most common and most severe seizures began with explosive jumping or running fits; this was then followed by barrel rolls and/or loss of postural control intermingled with clonic movement of all limbs; seizures usually terminated with tonic extensions of the forelimbs or tail. The convulsive seizures ranged in duration from approximately 5-15 seconds. A seizure was then followed by a long period of immobility. In general, the term ‘seizure’ used in this study refers to the previous description. However, there were some variations of this basic

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7 Due to the severity of these convulsions, I prefer to not show images of these events. However, videos are available upon request.
theme. In addition, these animals also exhibited moderate convulsive behaviors such as jerks and tremors.

Data obtained from 24-hour video monitoring revealed that convulsive activities occurred with a latency of 2.12±0.48 hours following HI, and the mean incidence of convulsions was 0.69±0.065 events per hour (n=20 mice). The overall convulsion severity was 3.88±0.16 as per a modified 0-5 scale previously established for primary generalized seizures (see Methods 3.3). In contrast, severe motor seizures and convulsive behavior were absent in the remaining 43 mice (57.6%) examined.

Furthermore, convulsions were not observed in 14 mice examined 1-4 weeks post HI. Of these 14 mice, 3 mice had exhibited severe motor seizures within 72 hours of HI. However, as video and EEG monitoring were intermittent, this approach may have failed to detect the occurrence of chronic or late-onset post-ischemic seizures in this group of animals.

For the remainder of this thesis, the term ‘seizure’ refers to animals exhibiting at least 2 vigorous seizures (stages 4-5, as described above). Furthermore, the terms ‘convulsive’, ‘motor’, or ‘behavioral’ are used interchangeably when applied to these seizures. To my knowledge, this is the first report of early post-ischemic convulsive seizures following HI in adult mice.

7.2 Post-HI seizures are associated with mortality and brain injury

In the following discussion, the term ‘seizure’ refers to animals exhibiting at least 2 vigorous motor seizures (stages 4-5, as described above).

An examination of the relationship between severe seizures and subsequent outcome revealed two important correlations.

Firstly, the occurrence of severe motor seizures is correlated to mortality. Mortality occurred in 21 of 33 (63.6%) mice with severe seizures but only in 4 of 43 mice (9.3%) without severe motor seizures. The rate of mortality was significantly greater in animals with seizures (p<0.001, Fisher Exact Test; Figure 23A). Thus, the occurrence of severe motor seizures is correlated to post-HI mortality; however, neither the mechanisms of post-HI mortality nor the mechanisms by which seizures contribute to post-HI mortality were directly examined in this study.
Secondly, there is a correlation between seizures and brain injury. Seizures were detected in 20 of 39 mice (51.3%) with clear ipsilateral brain injury but only in 1 of 23 (4.3%) mice with minimal/unclear brain injury. Thus, the probability of observing seizures was significantly higher in animals with clear ipsilateral brain injury than in animals with minimal/unclear brain injury (p<0.001, Fischer Exact Test; Figure 23B).

The above observation indicates that seizures are correlated to clear panhemispheric brain injury; to determine whether seizures are associated with increased brain injury, detailed histological assessments of ipsilateral infarct area were carried out in animals with clear ipsilateral brain injury that did (n=8) or did not exhibit (n=16) severe behavioral seizures. Animals with minimal/unclear brain injury were not included in this analysis. No significant differences were observed in the extent of ipsilateral brain tissue loss (relative to the contralateral hemisphere) in the 8 coronal planes examined (p=0.353; Two-Way ANOVA; Figure 23B). These observations suggest that the development of early-onset post-HI motor seizures is not associated with increased brain injury. However, as a caveat to interpreting the previous observation, it should be noted that only surviving animals were incorporated into the seizure group for analysis of infarct area at 6 weeks post-HI, and this may have influenced the results. Nonetheless, I suggest that acute seizures following HI are strongly associated with ipsilateral brain injury and mortality.
Figure 23. Early-onset seizures are closely associated with infarction and mortality

A

no seizure (n=43)

- 90.7%
- 9.3%

seizure (n=33)

- 63.6%
- 36.4%

☐ survival ☐ mortality

B

minimal/unclear injury
(n=23)

- 95.7%
- 4.3%

clear injury (n=39)

- 51.3%
- 48.7%

☐ without seizures ☐ with seizures
Figure 23. Early-onset seizures are closely associated with infarction and mortality

A, Post-HI seizures are associated with increased mortality. Mortality was encountered in 21 of 33 (63.6%) mice with severe seizures (right) but only in 4 of 43 mice (9.3%) without severe seizures (left). *, p<0.001; Fisher Exact Test. B, Post-HI seizures are associated with ipsilateral brain injury. Above, incidences of seizures in mice with or without clear brain injury. Seizures were detected in 20 of 39 mice (51.3%) with clear ipsilateral brain injury (right), and in 1 of 22 (4.4%) mice with minimal/unclear brain injury (left). *, p<0.001; Fisher Exact Test. Below, injury of the ipsilateral hemisphere was quantified at the 8 coronal planes in mice with clear ipsilateral brain injury with (n=8) or without (n=16) seizures. Ipsilateral brain injury was quantified as described in Figure 2, and data were expressed as % reduction in ipsilateral to contralateral hemispheric area. Only animals exhibiting clear ipsilateral brain injury were included for analysis. No significant differences were observed in the extent of ipsilateral brain tissue loss (relative to the contralateral hemisphere) in the 8 coronal planes examined (p=0.353; Two-Way ANOVA). Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
7.3 Treatments with anticonvulsants improve post-HI outcome

To further examine the relationship between seizures and post-HI outcome, animals were treated with a combination of diazepam (0.15mg/Kg) and phenytoin (18mg/Kg). The combination and dosages of this treatment are based on the protocol recommended for controlling generalized convulsive status epilepticus in clinical settings (Meierkord et al. 2010). Diazepam enhances the action of GABA on GABA\textsubscript{A} receptors, which therefore enhances inhibition; the mechanisms of action of phenytoin are not completely understood, but are likely due to the inhibition of voltage-gated sodium channels, which therefore reduces cellular excitability. Prophylactic treatment is not recommended for post-stroke seizures in humans; nonetheless, prophylactic treatment has been shown to be far more effective at controlling non-convulsive seizures that occur in rats following experimental occlusion of the middle cerebral artery (Williams et al. 2004; Williams et al. 2006). Thus, both drugs were administered prophylactically within 5 minutes following HI and prior to detectable motor seizures. In the following discussion, the term ‘seizure’ refers to animals exhibiting at least 2 vigorous motor seizures (stages 4-5, as described above).

Treatment with diazepam and phenytoin significantly reduced the proportion of animals exhibiting severe motor seizures within 72 hours following HI. Seizures were observed in 7 of 16 mice (43.7%) that received saline injections but only in 2 of 31 mice (6.4%) treated with diazepam and phenytoin (p=0.004; Fisher Exact Test; Figure 24A).

Moreover, the drug treatment significantly reduced mortality following HI. Mortality was encountered in 5 of the 16 mice (31.2%) that received saline injections (all 5 animals died overnight) and only in the two treated animals described above in which seizures were refractory to prophylactic treatment (both animals euthanized according to ethical guidelines) (p=0.036; Fisher Exact Test; Figure 24B).

However, the proportion of animals that developed clear ipsilateral brain injury was not significantly different between saline-injected (8 of 11 mice) and the drug-treated (16 of 31 mice) groups (p=0.299; Fisher Exact Test). In addition, the extent and magnitude of ipsilateral infarct area were similar between the saline-injected and drug-treated animals when examined at
8 coronal planes at 6 weeks post-HI (p=0.619; Two-Way ANOVA; Figure 24C). This result suggests that prophylactic treatments with anticonvulsant drugs do not alter infarct size\textsuperscript{8}.

Utilizing the same combination of diazepam and phenytoin, 8 mice were treated at the onset of the first observable seizure (within 3 hours post-HI). Seizures were transiently suppressed in all 8 animals, but were observed again within a period of 24-72 hours following the treatment. Mortality occurred in 7 of 8 mice (87.5\%) subjected to delayed treatment (data not illustrated). Given the high rate of mortality with delayed treatment, these experiments were aborted.

Taking these observations together, I suggest that prophylactic treatment with diazepam and phenytoin reduces the incidence of acute behavioral seizures and improves survival following HI, but is insufficient to greatly influence the extent of brain injury – although it remains to be determined whether anticonvulsants can influence the extent of early brain injury in the absence of a possible confounding effect by mortality. In general, these results are in accordance with previous studies utilizing phenytoin in neonatal rats following HI episodes (Vartanian et al. 1996) and in adult rats following focal ischemic episodes (Williams et al. 2004). However, other anticonvulsants and/or higher dosages of diazepam and phenytoin need to be tested to fully explore these issues.

\textsuperscript{8} Brain injury was examined at 6 weeks post-HI for two reasons: 1) To rule out the possibility of delayed injury that may have occurred as a consequence of treatment. 2) It is easier to quantify brain injury when large cystic infarcts are evident (this requires at least 1 week). However, delaying investigations for this period of time introduces complications of mortality in the control group, which may influence the results, as information of brain injury in animals that died is lost (these are presumably the most severely injured animals). Future investigations aimed at examining brain injury at early post-HI times, prior to mortality, should be carried out to fully examine these issues.
Figure 24. Treatment with anticonvulsants reduces seizure incidence and mortality

A

B

C

ipsilateral infarct area (%)
Figure 24. Treatment with anticonvulsants reduces seizures incidence and mortality

A and B, Mortality and seizure incidence in animals that received diazepam (DZ) + phenytoin (Phen) or saline injection. A, seizures were observed in 7 of 16 (43.7%) saline-injected animals and in 2 of 31 (6.4%) drug-treated animals. *, p=0.004; Fisher Exact Test. B, mortality was encountered in 5 of 16 (31.2%) saline-injected animals and in the 2 of 31 (6.4%) drug-treated animals described above in which seizures were not controlled. *, p=0.036; Fisher Exact Test. C, drug treatments do not affect the magnitude of ipsilateral brain injury. Above, representative images were obtained from 2 mice 6 weeks following saline or drug injection. The images were taken from coronal sections corresponding approximately to Bregma -1.5 mm. Note the extensive injury in the ipsilateral (right) hemisphere in both images. Below, detailed histological assessments were conducted 6 weeks post-HI for saline-injected (n=6) and drug-treated (n=12) animals. Ipsilateral brain injury was quantified as described in Figure 2, and data were expressed as % reduction in ipsilateral to contralateral hemispheric area. Only animals exhibiting clear ipsilateral brain injury were included for analysis. No significant group differences were observed at the 8 different coronal planes examined (p=0.619; Two-Way ANOVA). Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
7.4 Summary of chapter 7

Approximately 44% of mice exhibited convulsive motor seizures within 72 hours following HI. These seizures were severe, vigorous, and generalized in regard to behavioural manifestations. A statistically significant correlation was found between seizures and clear ipsilateral brain injury, as well as between seizures and subsequent mortality. Importantly, prophylactic treatment with a combination of anticonvulsant drugs reduced seizure incidence and mortality, but did not influence the extent of brain injury. Delaying treatment until seizures were observed was ineffective in reducing seizure incidence or mortality. To my knowledge, this is the first demonstration and examination of early motor seizures following HI in adult mice.
Chapter 8

8 Results V: EEG activity during post-HI seizures

The electroencephalogram (EEG) is a widely used diagnostic tool for detecting post-ischemic seizures in both animal models and humans (Jordan 2004; Kelly 2007). Thus, the objective of this section is to determine whether post-HI motor seizures are associated with electrographic discharges in the hippocampus and cerebral cortex.

8.1 Non-convulsive EEG discharges during HI

During the late stages of the hypoxic episode or within a few minutes following HI, one or two episodes of low-amplitude rhythmic discharges (~15-40 spikes per second, duration of 4-18 seconds) were observed in the ipsilateral hippocampus or cortex but not in the contralateral counterpart structures in 8 of 36 mice (22.2%) examined (Figure 25). Prior to, during, and following these discharges, the animals were completely immobile and displayed no evidence of convulsive behavior; that is, these discharges are non-convulsive. Furthermore, these discharges were observed on a strongly suppressed EEG background relative to baseline. It is worth noting that 7 of the 8 animals displaying such epileptiform discharges either died or demonstrated clear ipsilateral brain injury in later histological assessments.
Figure 25. Non-convulsive ipsilateral EEG discharges during and following HI

**A**

ipsilateral cortex

**B**

ipsilateral hippocampus
Figure 25. Non-convulsive ipsilateral EEG discharges during and following HI

**A and B**, low amplitude rhythmic discharges from the ipsilateral cortex and hippocampus, respectively, with shaded regions expanded below. Power spectra are shown on the right. One or two episodes of similar discharges were observed in the ipsilateral hippocampus or cortex but not in contralateral regions during and shortly following HI in 8 of 36 mice (22.2%) examined. Animals were completely immobile prior to and during these discharges; that is to say, these discharges are non-convulsive. It is worth noting that 7 of the 8 animals displaying such epileptiform discharges either died or demonstrated clear ipsilateral brain injury. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
8.2 Post-HI seizures are not associated with cortical and hippocampal EEG discharges

Surprisingly, no evident electrographic discharges were observed to precede or coincide with the severe motor seizures described above. Motor seizure events (>3 events per animal) were encountered during EEG recording sessions from the ipsilateral hippocampus (n=10 mice), ipsilateral entorhinal cortex (n=5 mice), ipsilateral parietal cortex (n=5 mice), contralateral hippocampus (n=2 mice), and contralateral parietal cortex (n=10 mice). However, in correlation with such vigorous motor convulsions, EEG signals were frequently contaminated with large artifacts (due to movement-related interference by recording cables), which might mask potential EEG discharges (Figure 26). Despite the obscuring of EEG recordings during such motor seizures, no evidence of interictal activity, ictal discharges, or postictal depression were noted. Moreover, ipsilateral EEG activity was profoundly suppressed relative to baseline prior to these behavioral convulsions.

In order to minimize movement artifacts during seizures, wireless telemetry EEG recordings were conducted. In 5 animals that exhibited severe seizures following HI, no EEG discharges were observed before and during motor seizures (Figure 27).
Figure 26. EEG activities associated with post-HI motor seizures with wired EEG recordings
Figure 26. EEG activities associated with post-HI motor seizures with wired EEG recordings

EEG recordings from a mouse with bilateral implanted electrodes in the ipsilateral hippocampus and contralateral cortex. EEG activity shortly prior to and at the start of a post-HI motor seizure is shown, with shaded regions expanded below. In correlation with vigorous post-HI motor seizures, EEG signals were frequently contaminated with large artifacts (due to movement-related interference by recording wires), which might mask potential EEG discharges. Despite the obscuring of EEG recordings during such motor seizures, no evidence of interictal activity, ictal discharges, or postictal depression were noted prior to or following the motor seizures in all mice examined. Notably, ipsilateral EEG activity was profoundly suppressed relative to baseline prior to these behavioral convulsions. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
Figure 27. Wireless telemetry recording during post-HI seizures reveals no EEG discharges.
Figure 27. Wireless telemetry recording during post-HI seizures reveals no EEG discharges

Ipsilateral cortical EEG activity was recorded via wireless radio telemetry in a post-HI mouse. Shaded region is expanded below. Telemetry recordings minimized movement related artifacts during severe motor seizures; however, in 5 animals that exhibited severe seizures following HI, no EEG discharges were observed before and during motor seizures. This example captured a period of a running fit. Modified from El-Hayek et al (2011a) with permission from Oxford University Press.
8.3 Summary of chapter 8

Non-convulsive ipsilateral EEG discharges were observed during and shortly following HI in 22% of mice; however, these discharges were not associated with behavioral convulsions. Thus, these discharges may represent a type of focal, non-convulsive epileptiform activity, resulting as a consequence of hypoxia-induced hyperexcitability. More importantly, no EEG discharges or aberrant EEG activity of any kind (except for profound suppression) was observed to precede or coincide with the severe convulsive motor seizures following hypoxia-ischemia in adult mice in hippocampal and cortical recordings. These observations suggest that post-HI motor seizures may originate in deeper subcortical structures.
Chapter 9

9 Results VI: Can EEG activity predict seizures?

The above findings indicate that severe post-HI motor seizures are not associated with electrographic discharges in the ipsilateral or contralateral forebrain regions examined. Despite the absence of electrographic discharges, I sought to determine whether intracranial EEG recordings from the ipsilateral hippocampus can herald the development of post-HI seizures. In the following description the term ‘seizure’ represents at least 2 severe motor seizures (stages 4-5) as described previously (see 7.1).

9.1 Profound EEG suppression in animals with seizures

EEG suppression has demonstrated utility in predicting outcome following HI with respect to brain injury and mortality (see chapter 6). Thus, given the close association of seizures with brain injury/mortality (see chapter 7), I retroactively sorted mice into two groups: mice that exhibited seizures and clear ipsilateral brain injury/mortality (n=9), and mice that did not exhibit seizures yet still demonstrated clear ipsilateral brain injury (n=7)\(^9\); and I compared the percent reduction (relative to baseline) in EEG amplitude, variance, and root mean square (RMS) between these groups of animals at the end of HI and at 1 hour post-HI (prior to the development of seizures)\(^10\) (Figures 26 and 27). These parameters have previously demonstrated effectiveness in predicting post-HI outcome (see chapter 6).

Both groups of animals demonstrated a significant reduction in EEG amplitude, variance, and root mean square (RMS) relative to sham control mice at the end of HI and at 1 hour post-HI (p<0.001, Two-way ANOVA, Student-Newman-Keuls test; Figures 29A). No significant group differences in EEG amplitude were observed at the two time points examined (p≥0.062, Two-way ANOVA, Student-Newman-Keuls test; Figure 29A). A significantly greater reduction in EEG variance was observed at 1 hour post-HI in mice that later developed seizures vs. seizure-
free animals (to 3.84±2.48% vs. 13.62±4.24% of baseline, respectively; p=0.005; Figure 29A), but not at the end of HI (p=0.122, Two-way ANOVA, Student-Newman-Keuls test). A significantly greater reduction in EEG RMS was observed in mice that later developed seizures vs. seizure-free animals both at the end of HI (to 12.63±3.02% vs. 37.64±10.25% of baseline, respectively; p=0.01) and at 1 hour post-HI (to 13.54±2.80% vs. 47.38±7.83% of baseline, respectively; p=0.002; Two-Way ANOVA, Student-Newman-Keuls test; Figure 29A).

Collectively, these observations indicate that the development of post-HI seizures is associated with a significantly greater suppression in EEG activity during and shortly following HI and that EEG RMS is a more sensitive measure of this suppression than EEG amplitude or variance.

9.2 Longer duration of EEG suppression in animals with seizures

As the suppression of synaptic activity is one the earliest responses to ischemia and anoxia in vivo and in vitro, the duration of EEG suppression during HI may thus indirectly represent sensitivity to ischemia and/or the duration of cerebral ischemia. Hence, I compared the duration of EEG suppression (~65% reduction in EEG variance relative to baseline) between the two groups of mice defined above. Animals that developed seizures exhibited a significantly longer duration of EEG suppression during HI compared to animals lacking seizures (18.3±2.2 min vs. 11.6±1.5 min, respectively; p=0.03; t-test; Figure 29B). Importantly, the duration of hypoxia was not significantly different between the two groups of animals (p=0.18; t-test; Figure 29B).

These observations suggest that animals that develop post-HI seizures exhibit increased sensitivity to EEG suppression during HI compared to animals lacking seizures.
Figure 28. EEG suppression in mice with or without post-HI motor seizures
Figure 28. EEG suppression in mice with or without post-HI motor seizures

Representative EEG traces were collected from 2 mice that were subjected to HI. Large amplitude events are movement artifacts. Traces were band-pass filtered (0.5 to 500Hz). Recordings were performed simultaneously from the ipsilateral hippocampal CA1 and contralateral cortex. Data segments were collected 7 days post-implantation (baseline), following occlusion of right common carotid artery (RCCA), during the entire course of the hypoxic episode, and at 1 hour post-HI. Lower traces are time-expanded segments. A, traces were obtained from a mouse that developed seizures post-HI. This mouse developed severe motor seizures approximately 3 hours post-HI and was euthanized at 48 hours post-HI according to ethical guidelines. B, traces were obtained from a seizure-free post-HI mouse. This mouse did not exhibit behavioral convulsions, neurological deterioration, and it survived up to 6 weeks post-HI at which point histological assessments revealed extensive brain injury in the ipsilateral hemisphere. Figure modified from EL-Hayek et al (2011b) with permission from Elsevier.
Figure 29. Quantification of EEG suppression in mice with or without post-HI seizures

A

variance

amplitude

RMS

% of baseline

end of HI 1 hour later

end of HI 1 hour later

end of HI 1 hour later

sham control (n=7 mice) seizure (n=7 mice) seizure (n=9 mice)

B

EEG suppression (min)

hypoaxia (min)

*
Figure 29. Quantification of EEG suppression in mice with or without post-HI seizures

Mice were sorted into seizure-exhibiting and seizure-free groups and EEG amplitude, variance, and RMS were compared at the end of HI and at 1 hour post-HI (prior to the development of post-HI convulsions). A, grouped EEG variance (left), amplitude (middle), and root mean square (RMS; left) were calculated and expressed as percent of baseline at the end of HI and 1 hour later. Statistically significant group differences are indicated. *, p≤0.01; Two-way ANOVA, Student-Newman-Keuls test. B, duration of EEG suppression and hypoxia during HI in mice that later developed seizures vs. seizure-free animals. Left, the duration of EEG suppression during the hypoxic episode (~65% reduction in EEG variance relative to baseline) was compared between the two groups of mice defined above. Animals that later developed seizures had experienced a longer duration of EEG suppression during HI. *, p=0.03; t test. Right, the duration of the hypoxic episode was not significantly different between the two groups of mice (p=0.18; t test). Figure modified from EL-Hayek et al (2011b) with permission from Elsevier.
9.3 Analysis of power spectral alterations in animals with seizures

To further determine whether EEG activity can predict post-HI seizures, I carried out an analysis of EEG frequency components. Dominant EEG frequency and associated power was analyzed at the end of HI and at 1 hour post-HI, and the data were compared between the two groups of animals defined above. Dominant frequency and associated power were compared in different spectral bands: delta (1-3.6Hz), theta (4-11.6Hz), beta (12-29.6Hz), gamma (30-100Hz), and high frequency (100.4-500Hz, HF) at the end of HI and within 1 hour post-HI. These frequency bands are based on previous studies of EEG activity in the rodent hippocampus (O’Keefe 2007).

No significant differences were observed with respect to dominant frequencies between the two groups of animals at baseline, during HI, or at 1 hour post HI in the delta (p=0.876), theta (p=0.433), beta (p=0.414), and gamma bands (p=0.967; Two-way ANOVA; Figure 30).

However, in animals that later developed seizures a significant increase in the dominant HF peak was noted during HI compared to animals that did not develop seizures (to 223.6±27.5Hz 149.0±26.3Hz; p=0.023; Two-Way ANOVA; Student-Newman-Keuls test test; Figure 30) However, no significant difference was noted at 1 hour post-HI (p=0.206). These observations indicate an increase in high frequency activity during HI is a potential predictor of the development of post-HI seizures. Further studies and/or more sophisticated analyses are required to fully elucidate this finding.

With respect to power, animals that exhibited seizures demonstrated a more profound suppression of peak delta power compared to animals lacking seizures during HI (to 4.89±2.41% vs. 50.4±24.02% of baseline, respectively; p=0.002) and at 1 hour post-HI (to 5.03±2.23% vs. 38.11±13.89% of baseline, respectively; p=0.007; Two-Way ANOVA; Student-Newman-Keuls test; Figure 31). A significant reduction in gamma power during HI was observed in animals that developed seizures compared to animals that did not develop seizures (to 1.32±0.64% and 4.83±1.79% of baseline, respectively; p=0.045), but not at 1 hour post-HI (p=0.14; Two-Way ANOVA; Student-Newman-Keuls test; Figure 31). Moreover, no significant differences in EEG power were observed in the theta (p=0.842), beta (p=0.457), or HF (p=0.853) bands (Two-Way ANOVA); however, larger sample sizes are required to fully examine the latter negative results.
Figure 30. Dominant EEG frequencies in animals with or without post-HI seizures

- Delta frequency
- Theta frequency
- Beta frequency
- Gamma frequency
- High frequency

- Seizures +injury/mortality (n=9 mice)
- No seizures + injury (n=7 mice)
Figure 30. Dominant EEG frequencies in animals with or without post-HI seizures

Mice with clear ipsilateral brain injury were sorted according to the observance of severe motor seizures or the lack thereof. Dominant frequency was compared in different spectral bands: delta (1-3.6Hz), theta (4-11.6Hz), beta (12-29.6Hz), gamma (30-100Hz), and high frequency (100.4-500Hz, HF) at the end of HI and within 1 hour post-HI. Data was analyzed at the end of HI and within 1 hour post-HI. *, p=0.023; Two-Way ANOVA, Student-Newman-Keuls test.
Figure 31. EEG dominant power in animals with or without post-HI seizures

- **Delta power**
  - % of baseline
  - Symbols: * indicates significant change.
  - Data points: end of HI, 1 hour later.

- **Theta power**
  - % of baseline
  - Symbols: * indicates significant change.
  - Data points: end of HI, 1 hour later.

- **Beta power**
  - % of baseline
  - Symbols: * indicates significant change.

- **Gamma power**
  - % of baseline
  - Symbols: * indicates significant change.

- **HF power**
  - % of baseline
  - Symbols: * indicates significant change.

Legend:
- **Black bars**: seizures + injury/mortality (n=9 mice)
- **Gray bars**: no seizures + injury (n=7 mice)
Figure 31. EEG dominant power in animals with or without post-HI seizures

Mice with clear ipsilateral brain injury were sorted according to the observance of severe motor seizures or the lack thereof. Peak power was compared in different spectral bands: delta (1-3.6Hz), theta (4-11.6Hz), beta (12-29.6Hz), gamma (30-100Hz), and high frequency (100.4-500Hz, HF). Data was analyzed at the end of HI and within 1 hour post-HI and expressed as percent of baseline. *, p≤0.007; Two-Way ANOVA, Student-Newman-Keuls test.
Collectively, the above observations indicate that animals that subsequently developed seizures exhibited a more profound suppression of EEG activity that is mostly due to suppression in the delta range, and this suppression is also associated with an increase in high frequency activity.

**9.4 EEG suppression in animals without post-HI seizures**

The above analyses indicate that when animals with clear ipsilateral brain injury are grouped according to the observance of seizures, animals with seizures exhibited a relatively more profound suppression in EEG activity. Yet an obvious question remains regarding EEG activity in animals with clear ipsilateral brain injury but lacking seizures: is there any difference in the extent of EEG suppression between these seizure-free mice and those described previously with minimal/unclear ipsilateral brain injury post-HI (see chapter 6)?

To address this issue, I examined the percent change of EEG RMS in three groups of animals: sham control mice (n=7), mice with minimal/unclear brain injury post-HI (n=14), and mice exhibiting brain injury post-HI but without seizures (n=7 mice). Interestingly, the magnitude of EEG suppression during HI was not significantly different between the mice with minimal/unclear brain injury and mice with clear brain injury without seizures during HI (55.37±10.90% vs. 37.64±10.25% of baseline, respectively; p=0.240) and at 1 hour post-HI (66.92± 6.98% vs. 47.38± 7.83% of baseline, respectively; p=0.196), though both groups were significantly suppressed with respect to sham controls (p≤0.038; Two-way ANOVA; Student-Newman-Keuls test; Figure 32). However, by 1 day post HI and thereafter, the EEG RMS in animals with minimal/unclear brain injury had recovered to control values (p≥0.276). In contrast, EEG RMS remained significantly suppressed in animals with clear ipsilateral brain injury yet lacking seizures for up to 6 weeks post-HI relative to sham controls and animals lacking brain injury (p≤0.033; Two-way ANOVA; Student-Newman-Keuls test; Figure 32).

These observations indicate that although the extent of EEG suppression during and shortly after HI is similar in animals with minimal/unclear brain injury and in animals with clear brain injury but without seizures, EEG suppression in the former group recovers by 1 day post-HI, while EEG suppression does not recover in the latter group during the course of the recording period. However, further experiments are required to fully address the lack of statistical significance during HI and at 1 hour post-HI.
Figure 32. EEG suppression in animals lacking post-HI seizures

**ipsilateral EEG RMS (% of baseline)**

- **end of HI**
- **1 hour**
- **1 day**
- **1 week**
- **4-6 weeks**

**Legend**
- **sham control** (n=7 mice)
- **minimal/unclear injury** (n=14 mice)
- **injury + no seizure** (n=7 mice)
Figure 32. EEG suppression in animals lacking post-HI seizures

Mice were sorted into three groups: sham control mice, mice with minimal/unclear brain injury post-HI, and mice with clear brain injury post-HI but without seizures. EEG root mean square (RMS) was calculated and is expressed as percent of baseline at the end of HI and at later times. Statistically significant group differences are indicated. Note that EEG activity in mice that exhibited clear brain injury without seizures remained significantly decreased throughout the recording period. *, p≤0.033; Two-way ANOVA; Student-Newman-Keuls test.
9.5 Summary of chapter 9

Although clear EEG discharges were not observed during post-HI motor seizures, EEG activity still demonstrates utility in predicting seizure genesis in post-HI animals. When animals with clear ipsilateral brain injury were sorted according to the observation of seizures (or the lack thereof), animals with seizures exhibited a more profound suppression of ipsilateral EEG activity and an increased sensitivity to ipsilateral EEG suppression during HI. Power spectral analysis reveals that animals with seizures exhibited an increase in high frequency activity during HI and a more profound suppression of delta power. These observations suggest that animals with seizures exhibit a greater sensitivity to EEG suppression during and following HI, and that an examination of EEG suppression is a useful parameter for predicting post-HI seizures despite the lack of observed EEG discharges from the same recording sites.
Chapter 10

10 Discussion

I have reported that HI episodes induce severe, early-onset convulsive seizures in adult mice, that these post-HI seizures are correlated to profound, lateralized cerebral ischemia, and that they are associated with post-HI mortality.

There are, in my opinion, four major issues that require further discussion:

1) How does HI result in ipsilateral brain injury?

2) Why does EEG activity predict post-HI brain injury, motor seizures, and mortality?

3) Why are post-HI motor seizures associated with mortality and brain injury?

4) Why are post-HI motor seizures not associated with electrographic discharges?

10.1 Modes of post-HI brain injury

Extensive ipsilateral brain injury following HI was observed in approximately 27 of 38 animals (71%) subjected to detailed histological assessments. In general, brain injury was consistently observed in brain regions supplied by the three major cerebral arteries, including large portions of the cerebral cortex, hippocampus, thalamus, and basal ganglia. In addition, mortality within 72 hours post-HI was encountered in 26 of 84 mice (31%). Overall, the observed pattern of brain injury, the proportion of animals developing clear brain injury, and the incidence of mortality are in accordance with previous reports (Vannucci et al. 2001; O'Donnell et al. 2002; Olson and McKeon 2004; Adhami et al. 2006). This validates the experimental paradigm under my conditions and further supports the reproducibility of the HI model in adult mice.

As described by others (Olson and McKeon 2004; Adhami et al. 2006), a proportion of animals did not exhibit clear ipsilateral brain injury post-HI in histological assessments. The development of severe brain injury initially appeared to be an ‘all-or-none’ phenomenon: individual mice
either exhibited clear panhemispheric brain injury or were indistinguishable from sham controls. However, these assessments were based on qualitative observations of cresyl violet stained sections; such qualitative assessments would not detect subtle brain injury. Therefore, to assess the possibility of subtle brain injury in these animals, TUNEL and FluoroJade staining were implemented. These markers are better suited to detect degenerating cells, and they were assessed at 72 hours post-HI in animals that lacked clear brain injury in cresyl violet stained sections. Interestingly, TUNEL and FluoroJade positive signals were observed in animals lacking clear ipsilateral brain injury in cresyl violet-stained sections, yet the density and uniformity of these signals were highly variable, and were relatively lower when compared to animals that exhibited clear brain injury in cresyl violet-stained sections. These results indicate that a subset of post-HI mice may exhibit subtle brain injury, characterized by sparse cell loss, which may escape detection by standard histological assessments. Consequently, this mode of ipsilateral brain injury was referred to as ‘minimal/unclear’ brain injury. To my knowledge, this is the first description of a subtle mode of brain injury in mice post-HI. However, future examinations are required to characterize this issue in depth and to reveal underlying mechanisms and functional consequences.

10.2 Possible mechanisms of HI-induced brain injury

Despite the observation of subtle brain injury described above, my results clearly demonstrate that some animals suffered a catastrophic panhemispheric infarction while others did not. Although this dichotomy is a well-known characteristic of the HI model (Vannucci et al. 2001; O'Donnell et al. 2002; Olson and McKeon 2004; Adhami et al. 2006; Kadam et al. 2010), the underlying mechanisms for this variability have not been effectively addressed. This is an interesting and complex issue, and it speaks directly to the mechanism of brain injury in this experimental ischemic model.

HI is a two-step model: it combines unilateral ligation of the common carotid artery with systemic hypoxia. It is well established that unilateral ligation of the right common carotid artery (RCCA) alone does not result in appreciable brain injury in adult C57BL/6 mice and rats, although white matter lesions to the corpus callosum have been described (Olson and McKeon 2004; Yoshizaki et al. 2008). In addition, my results indicate that RCCA ligation alone (in the absence of respiratory hypoxia) does not result in alteration or suppression of intracranial
electroencephalographic (EEG) activity recorded from the ipsilateral hippocampus in C57BL/6 mice (see Appendix II). Moreover, transient respiratory hypoxia alone does not result in brain injury in rodents (Jensen et al. 1992; Vannucci et al. 2001; Olson and McKeon 2004; Adhami et al. 2006; Wais et al. 2009). Yet the combination of RCCA ligation and respiratory hypoxia can result in massive panhemispheric brain damage, and the mechanisms for this additive effect are not clear.

Normal cerebral blood flow (CBF) is approximately 55 mL · 100 g⁻¹ · min⁻¹; and, in most species studied, including humans, unless blood flow is reduced below a flow rate of 10-12 mL · 100 g⁻¹ · min⁻¹ for a significant period of time, infarction will not occur (Howells et al. 2010). Utilizing laser Doppler-based measurements of cortical blood flow, I (see 5.5) and others (Adhami et al. 2006; Todo et al. 2008; Yoshizaki et al. 2008; Meng et al. 2009) have demonstrated that RCCA ligation results in a ~50% reduction in cortical CBF, and this does not appear to be sufficient to result in brain infarction in C57BL/6 mice. This supports previous studies that have suggested that regional CBF must decline to <25% of baseline for a sufficient period of time to result in a high probability of infarction (Ginsberg 2003; Adhami et al. 2006). Thus, RCCA ligation in mice is insufficient to result in extensive stroke-like brain injury, presumably due to a combined effect of collateral circulation via the circle of Willis and autoregulation of the cerebral vasculature, both of which can maintain sufficient flow rates in response to varying cerebral perfusion pressures (see Appendix I).

Thus, as RCCA occlusion alone or respiratory hypoxia alone does not result in extensive brain injury, there are three major questions that need to be addressed regarding the modes and patterns of post-HI brain injury.

1. How does combining unilateral occlusion of the right common carotid artery with respiratory hypoxia result in brain injury?

2. How does HI result in panhemispheric infarction; that is, how does HI result in extensive brain injury throughout large regions of the ipsilateral hemisphere including regions supplied by the anterior, middle, and posterior cerebral arteries?

3. Why are some mice resistant to panhemispheric infarction following HI?
10.2.1 How does combining unilateral occlusion of the right common carotid artery with respiratory hypoxia result in brain injury?

The mechanisms of HI-induced brain injury are not clear. In the following pages I will speculate as to the possible causes. As described above, my results indicate that permanent RCCA ligation results in a ~50% reduction in ipsilateral cerebral blood flow (CBF). Others have demonstrated that this ~50% decline plateaus following the ligation and remains at a stable, unchanged level for up to 3 months (Adhami et al. 2006; Todo et al. 2008; Yoshizaki et al. 2008; Meng et al. 2009). I have also demonstrated that superimposing respiratory hypoxia (8% O₂) on the RCCA occlusion results in a further secondary decline in CBF, such that ipsilateral CBF is reduced by ~90% in animals that developed clear brain injury. This finding is in accordance with previous studies that have examined CBF alterations during HI in mice (Adhami et al. 2006) and rats (Fowler et al. 2003). This therefore suggests that respiratory hypoxia is a causal factor in reducing CBF during HI. Thus, HI results in brain injury through a two-step process: 1) mild hypoperfusion induced by RCCA ligation and 2) an unknown mechanism of hypoxia-induced secondary cerebral ischemia/oligemia. Together these two factors reduce CBF below a threshold sufficient to result in brain injury.

However, an obvious question naturally follows from this reasoning:

How does respiratory hypoxia when superimposed on RCCA ligation result in a further decline in cerebral blood flow?

It should be noted that this result is, in my opinion, not directly intuitive. I will thus propose three speculative responses to address this question and, by inference, I will address question 1 posed above.

Response 1: Respiratory hypoxia when superimposed on RCCA ligation results in a further decline in CBF because of reduced mean arterial blood pressure.

It has been demonstrated that hypoxia superimposed on unilateral ligation of the common carotid artery results in a 50-70% reduction in mean arterial blood (MAP) pressure in rats and mice, and this is correlated to a pronounced reduction of CBF in ipsilateral hemispheres (Vannucci et al.
Fowler et al. (2006) have therefore concluded that a reduction in MAP may be responsible for the decline in CBF observed during hypoxia. The mechanisms for this decline in MAP are not clear. As hypoxia is a potent vasodilator, systemic vasodilatation may lower peripheral vascular resistance thus lowering MAP. However, a precise correlation of MAP to subsequent brain injury has not been carried out. Nonetheless, this is a plausible hypothesis that has experimental support.

Response 2: Respiratory hypoxia when superimposed on RCCA ligation results in a further decline in CBF because of rapid intravascular coagulation.

Another possible explanation for the decline in CBF may be due to rapid hypoxia-induced intravascular coagulation. Adhami et al. (2006) demonstrated that CBF does not recover during reoxygenation following HI. They further noted that reversing the RCCA ligation following the hypoxic episode does not result in a return of CBF to pre-HI levels (although reversible RCCA occlusion in the absence of a hypoxic episode results in a rebound of CBF to baseline levels). That is to say, the secondary ischemia/oligemia induced by respiratory hypoxia is irreversible, while the decline in CBF as a consequence of RCCA occlusion alone is fully reversible. Their explanation is that HI results in fibrin deposition in the cerebral microvasculature as early as 10 minutes following the hypoxic episode. The mechanisms for this rapid intravascular coagulation effect are not clear, and may be due to a direct action of hypoxia on the vascular endothelial cells. They further suggested that acute intravascular coagulation would lead to reperfusion deficits that would extend past the hypoxic episode; and that this “no reflow” phenomenon could result in long-lasting cerebral ischemia and exacerbate brain injury. To further support this hypothesis, they investigated the effects of HI on fibrinogen-null transgenic mice. Accordingly, while wild-type mice exhibited persistent reperfusion deficits following HI, fibrinogen-null mice experienced a rapid rebound in CBF upon reoxygenation; and this was correlated to reduced brain injury and lower mortality in these transgenic mice. However, it is interesting to note that fibrinogen-null mice still exhibited a substantial decline in CBF during the hypoxic episode that was comparable to that observed in wild-type mice. This indicates that although intravascular coagulation may play a role in reperfusion deficits, it cannot explain the decline in CBF during HI, although this issue was not discussed by the authors. Furthermore, it is unclear why pharmacological anticoagulants were not employed in their study to supplement the data from transgenic mice. Thus, the question of how CBF declines during HI still stands.
**Response 3:** Respiratory hypoxia when superimposed on RCCA ligation results in further declines in cerebral blood flow because vascular autoregulation is compromised.

Another possibility is a direct or indirect effect of hypoxia on the cerebral vasculature by a disruption of vascular autoregulation or neurovascular coupling. Through reasons that are not well-understood, the cerebral arterial vasculature is capable of maintaining an approximately constant blood flow to the brain in the face of changes in cerebral perfusion pressure, and this is brought about by vasodilation or vasoconstriction. This is referred to as autoregulation (Ganong 2001). Neurovascular coupling, on the other hand, refers to the increase in local cerebral blood flow to match energy demands, and is brought about by the release of vasodilating paracrines and metabolites from neurons and astrocytes (Attwell et al. 2011).

Adhami et al. (2006) have demonstrated that the partial pressure of oxygen in arterial blood sampled from the contralateral common carotid artery (CCA) during HI is reduced to 40 mmHg, with a corresponding 60% decrease in oxygen saturation. Furthermore, increases in the partial pressure of arterial blood CO$_2$ and acidosis were also observed. Low oxygen, elevated CO$_2$, and acidosis are potent vasodilators, and this would be predicted to increase rather than decrease CBF in the ipsilateral hemisphere. It should be noted, however, that sampling arterial blood from the contralateral CCA does not necessarily correlate to the tissue level, particularly in a hypoperfused ipsilateral hemisphere. A resolution of this issue would require that brain tissue oxygen measurements be carried out in conjunction with more detailed assessments of CBF, such as functional magnetic resonance imaging and high resolution assessments of vasodilation/vasoconstriction of the cerebral vasculature.

However, although low oxygen partial pressure is a potent vasodilator, extremely low oxygen partial pressures can indirectly interfere with cerebrovascular autoregulation and/or neurovascular coupling by triggering cortical spreading depression in brain tissue, which is a self-propagating wave of depolarization that is strongly implicated in ischemic/anoxic brain injury (Somjen 2001; Somjen 2004; Shin et al. 2006; Dreier et al. 2009; Lauritzen et al. 2011; see 10.3). Importantly, spreading depression can result in altered CBF dynamics and long-lasting alterations in cerebrovascular autoregulation (Lauritzen et al. 2011). For example, in an elegant study, Shin et al (2006) demonstrated that the decline in CBF following middle cerebral artery occlusion (MCAO) in rats can be divided into two phases: MCAO results in a rapid primary
decline in CBF and this is then followed a few minutes later by a secondary decline in CBF that is temporally coincident with cortical spreading depression. It is tempting to draw parallels between these observations and the two-stage declines in CBF I observed during HI. The mechanisms by which cortical spreading depression reduces blood flow under these conditions are unclear, but are speculated to be a consequence of arterial/arteriolar vasoconstriction due to direct depolarization of vascular smooth muscle cells resulting in contraction, an inhibition of nitric oxide synthase (nitric oxide is a potent vasodilator), and a release of local vasoconstrictive metabolites, such as arachidonic acid, from astrocytes (Somjen 2004; Attwell et al. 2010; Lauritzen et al. 2011). Future studies aimed at examining the onset of cortical spreading depression and the potential vasoconstriction of the cerebral vasculature during HI are necessary to examine this hypothesis. Experiments to address this hypothesis are discussed in chapter 11 (see 11.3).

10.2.2 How does HI result in panhemispheric brain injury?
A response to this question will require an in depth technical explanation of the cerebral vasculature and collateral pathways of cerebral blood flow. These issues are extensively discussed in Appendix I. A brief and general explanation is given here, and I refer the interested reader to Appendix I for more information.

HI results in brain injury throughout the ipsilateral hemisphere across regions supplied by all three cerebral arteries: the anterior, middle, and posterior cerebral arteries. In rodents and humans, the internal carotid artery branches into the middle and anterior cerebral arteries (Scremin 2004a, Scremin 2004b); thus, unilateral occlusion of the common carotid artery is expected to result in a decline in CBF in brain regions supplied by these arteries. A more pressing issue, however, is with the posterior cerebral artery, which, in humans, normally derives most of its blood from the vertebrobasilar system. The posterior cerebral arteries supply regions such as the medial temporal/occipital lobes and the hippocampus. In humans, blockade of the internal carotid artery at its bifurcation (at the so called carotid T) will not result in infarction in the territory of the posterior cerebral artery unless there is a congenital anatomical defect referred to as a fetal-type posterior cerebral artery; in this case, the posterior cerebral artery derives most of its blood from the internal carotid artery (van Raamt et al. 2006). The circle of Willis in mice and rats closely resembles this anatomical configuration (Barone et al. 1993; Kitagawa et al.
1998; Ozdemir et al. 1999; McColl et al. 2004; Scremin 2004b; Zhen and Doré 2007); thus, ligation of the common carotid artery is expected to result in a pronounced decline of CBF to brain regions supplied by the posterior cerebral artery. Thus, as regions supplied by the ipsilateral anterior, middle, and posterior cerebral arteries are expected to be hypoperfused following RCCA ligation, superimposing respiratory hypoxia may then result in panhemispheric brain injury according to any of the hypotheses listed above in response to question 1.

10.2.3 Why are some animals resistant to panhemispheric infarction following HI?

My data based on laser Doppler measurements of cerebral blood flow (CBF) indicates that animals that subsequently developed panhemispheric infarcts experienced a significantly greater decline in CBF compared to animals with minimal/unclear brain injury. This suggests that animals with minimal/unclear brain injury experienced a relatively milder ischemic insult.

There is extensive intra-strain variability in the cerebral vasculature within and across mice strains, including the C57BL/6 mouse, and these anatomical differences have been implicated in variable outcome following experimental cerebral ischemia due to variability in the extent of collateral blood flow (Barone et al. 1993; Kitagawa et al. 1998; Ozdemir et al. 1999; McColl et al. 2004; Scremin 2004b; Zhen and Doré 2007; see Appendix I). These anatomical differences may explain the resistance of some mice to HI-induced cerebral ischemia. However, if variability in compensatory collateral flow is a factor, then it may be inferred that RCCA ligation (prior to hypoxia) will result in more severe declines of CBF in animals that later developed extensive brain injury relative to animals with minimal/unclear brain injury. In my experiments, the CBF decline caused by RCCA ligation alone was not significantly different between animals that did or did not develop extensive brain injury post-HI. However, as only a small number of animals were examined, future experiments are necessary to fully examine this possibility.

10.3 EEG activity as a marker for post-HI outcome

To assess HI-induced changes in synaptic function, both chronic intracranial recordings in free-moving mice in vivo and electrophysiological recordings in brain slices in vitro were carried out. The suppression of electroencephalographic (EEG) activity is believed to reflect the extent of cerebral ischemia in animal models (Raffin et al. 1991; Lu et al. 2001; Hartings et al. 2003; Tichauer et al. 2009; Bjorkman et al. 2010; Barth and Mody 2011) and humans (Jordan 2004;
Friedman et al. 2009; Friedman et al. 2010, and EEG amplitude is employed clinically as a parameter to assess outcome following hypoxic-ischemic encephalopathy in neonatal humans (Shalak et al., 2003; Tao and Mathur, 2010). The advantage of the EEG in experimental studies of cerebral ischemia is that it can be used continuously in non-anesthetized animals, theoretically in any given brain region, and may be potentially employed as an in vivo and ‘online’ indicator of brain injury and treatment efficacy. A reliable and reproducible experimental model of EEG dysfunction following cerebral ischemic episodes would be beneficial in examining the role of the EEG in predicting outcome and the utility and comparison of quantifiable EEG parameters, in addition to investigations of treatment strategies on ongoing brain activity. However, experimental models of cerebral ischemia are potentially confounded by a reliance on anesthesia and/or long, complex surgeries (Hossmann, 2007), and this may influence the timing and extent of ischemia-induced EEG alterations. Moreover, the quantification and utility of EEG activity in the context of cerebral ischemia remains to be standardized in both clinical and experimental practice.

The electrophysiology of cerebral ischemia and anoxia has been extensively studied both in vivo and in vitro (Somjen 2001; Krnjević 2008). Typically, anoxia and/or ischemia results in a rapid suppression of synaptic transmission. The initial suppression of synaptic transmission is mainly attributed to the failure of presynaptic neurotransmitter release. Importantly, this initial suppression in synaptic transmission is fully reversible provided that oxygen or blood flow is promptly reintroduced: this may be a protective mechanism to reduce the metabolic demands of neural tissue during ischemia or anoxia. However, if ischemia or anoxia is prolonged, a reduction in oxidative phosphorylation leads to reduced cellular ATP, leading to the failure of the cell membrane Na⁺-K⁺ pump, and a build-up of extracellular K⁺. This consequently leads to cortical spreading depression. In the context of ischemia or anoxia, cortical spreading depression is referred to as hypoxic spreading depression, anoxic depolarization, ischemic depolarization, or terminal depolarization (Somjen 2001; Krnjević 2008). This event is a propagating, self-generating wave of depolarization that travels through brain tissue at a rate of 2-5 mm/min. Spreading depression results in a further increase in extracellular K⁺ up to 50 mM at the wavefront and severe depolarization, consequently leading to massive influx of Na⁺ and Ca²⁺ in combination with the release of excitotoxic amounts of glutamate (primarily through the reversal of glutamate transporters). This results in cytotoxic edema, mitochondrial damage, and the
activation of a number of Ca\textsuperscript{2+}-sensitive lipases and proteases, leading to irreversible structural damage to cells (Lipton 1999). In this case, the suppression in synaptic transmission will be permanent and irreversible.

My results with intracranial EEG recordings agree with the above sequence of events. A severe and permanent suppression of EEG activity was noted in animals with clear brain injury/mortality, while animals with minimal/unclear brain injury demonstrated a relatively milder and reversible suppression in EEG activity. Unfortunately, the experimental paradigm employed precluded an investigation of cortical spreading depression. The electrophysiological hallmark of cortical spreading depression is a large-amplitude, negative DC shift in the extracellular potential. In my experiments, EEG signals were low-pass filtered, which does not allow for a proper investigation of DC shifts\textsuperscript{11}. Future investigations are required to resolve and confirm the occurrence of cortical spreading depression during HI.

My results indicate that both the magnitude of EEG suppression and the disruption of intrinsic brain rhythms are good indicators for long-term assessments of brain injury following HI insults in mice. EEG amplitude, variance, and root mean square (RMS) were significantly reduced during and following HI by a greater magnitude in animals that subsequently developed clear ipsilateral brain injury compared to animals with minimal/unclear brain injury. Moreover, detailed examinations of EEG suppression can also predict the development of subsequent post-HI seizures and mortality. Interestingly, the RMS of the EEG power spectrum demonstrated the greatest sensitivity in predicting the development of post-HI seizures and mortality. The RMS is based on the decomposition of the EEG signal into its frequency components via the discrete Fast Fourier Transform (FFT). The RMS can therefore be roughly thought of as mean amplitude in the frequency domain, while EEG amplitude and variance can be thought of as measurements of mean amplitude in the time domain. The greater sensitivity of this parameter over EEG

\textsuperscript{11} The DC component was filtered for three-reasons. Firstly, cable-artifacts induced during movement or grooming would result in large-amplitude shifts in the EEG signal (in some cases, out the digitizer’s range). Filtering the DC component helped mitigate the effects of these artifacts. Secondly, EEG signals were amplified 1000 times prior to digitization. At such high amplification, any drift in the DC potential at the electrodes, for example a DC potential produced by the liquid interface with the wires, would be amplified and may saturate the digitizer. Thirdly, the previous explanation also applies to DC shifts associated with spreading depression, which can reach tens of millivolts.
amplitude and variance indicates that there is additional ‘information’ in the frequency domain, and supports the use of FFT-based approaches in assessing outcome following cerebral ischemia.

Adhami et al (2007) demonstrated that clear hippocampal injury was not evident in histological assessments until 12 hours post-HI, and that break down of the blood brain barrier did not occur until 24 hours post-HI. As my results indicate that early alterations in EEG activity within 1 hour post-HI can distinguish between post-HI outcomes, it therefore follows that EEG activity is a temporally sensitive marker for the development of subsequent ischemic brain injury, seizures, and mortality. This further supports the viewpoint that synapses are the first targets of HI, and that magnitude of the suppression of synaptic activity and its recovery (or lack thereof) are rapid indicators of subsequent infarction and mortality following cerebral ischemia (Somjen 2001).

Indeed, others have attempted such an approach in other experimental stroke models (Raffin et al. 1991; Hartings et al. 2003; Lu et al. 2001). However, my study has several advantages over previous approaches: 1) EEG activity was monitored in non-anesthetized free-moving animals. Brain EEG activity is radically altered by anesthesia, and anesthetics can have complex long-term effects on the brain physiology and can influence the outcome following experimental cerebral ischemia (Krnjević 1992; Rammes et al. 2009; Hallows et al. 2010). This may complicate results and interpretation. 2) In my experiments, mice were examined for a longer period lasting up to 6 weeks, such that long-term effects on EEG activity were monitored. 3) EEG activity was examined in different behavioral states utilizing several different parameters. 4) No animals were excluded from EEG analyses; hence the potential utility of the EEG to distinguish outcome was effectively examined.

The data presented here are the first characterization of the effects of HI on EEG activity in adult mice, and, in my opinion, represent one of the most – if not the most– detailed investigations of long-term EEG activity following experimental cerebral ischemia in rodents. The results further demonstrate that HI is a useful experimental model for assessing any concerns on the feasibility or utility of EEG recordings in an experimental setting.
10.4 Main features and pathological significance of post HI seizures

Previous studies have reported the occurrence of both non-convulsive and convulsive seizures in adult animals following focal cerebral ischemia (see Chapter 1, 1.5); however, a detailed examination of post-ischemic convulsive motor seizures is lacking. Using HI as a model for cerebral ischemia in adult mice, this study has provided novel insights and an overall characterization of post-ischemic convulsive seizures. The observations can be summarized into five main points: 1) Seizures are early-onset, occurring largely within 24 hours post-HI. 2) They are generalized motor seizures. 3) Generation of these seizures is associated with extensive brain injury because the seizures occur nearly exclusively in animals with extensive brain injury but not in animals with minimal/unclear injury. 4) These seizures are significantly associated with acute mortality and post-HI mortality is reduced by anticonvulsants. 5) These seizures are not associated with ictal EEG discharges in recordings from the ipsilateral or contralateral hippocampus or cerebral cortex.

Thus, based on the above data, I hypothesize that the early-onset, convulsive seizures are a major pathological component in the mouse model of HI. Furthermore, the strong association of post-HI seizures with brain injury is similar to the recently advanced concept of “no lesion, no epilepsy”, regarding the development of late-onset epileptogenesis following HI in neonatal rats (Kadam et al. 2010). However, whether seizures are causal or incidental in the process of ischemic brain injury remains to be elucidated. Nonetheless, I thus suggest that both early and late-onset seizures following ischemic episodes are intimately associated with the process of ischemic brain injury and mortality.

10.5 Mortality, brain injury, and seizures

The results of this study have indicated that HI can induce substantial mortality. The mechanisms of post-HI mortality were not examined in this study nor have they been examined by others. However, drawing parallels to large hemispheric stroke in humans may be useful. In humans, acute occlusion of the cervical portion of the internal carotid artery can result in large hemispheric stroke and panhemispheric infarction, which results from ischemia in the territories of the three cerebral arteries (Steiner et al. 2001; Schwarz et al. 2001; Kumral et al. 2009). Mortality rates following large hemispheric stroke can be as high as 80%, and the main causes of
mortality and neurological deterioration are believed to be increased intracranial pressure (ICP) and tissue shifts due to swelling of the brain (cerebral edema).

Cerebral edema following ischemia has two main components (Somjen 2004). Firstly, cytotoxic edema, or intracellular edema, occurs as a consequence of ionic imbalances and metabolic failure. In neurons, water is drawn into cells as a consequence of ischemic depolarization and extensive influx of Na\(^+\) and also by Gibbs-Donnan forces\(^{12}\). In the absence of cellular ATP to fuel active transport to restore ionic gradients, cells will swell. In addition, uptake of KCl can also cause swelling of astrocytes. Secondly, and more importantly, vasogenic edema, or extracellular edema, results as a consequence of damage to the capillary endothelium and a breakdown of the blood brain barrier (BBB). Once the BBB is damaged, it becomes permeable to osmotically active solutes, notably plasma albumin, which raises the colloid osmotic pressure of the interstitial fluid and draws water into the extracellular space, consequently resulting in swelling of the brain.

According to the Monro-Kellie doctrine, as the brain is enclosed by an osseous boundary, an increase in brain tissue volume due to edema must be compensated by a decrease in the volume of either intravascular blood or cerebrospinal fluid (Schwarz et al. 2001). The latter two account for approximately 20% of intracranial contents, with brain tissue accounting for the remainder. Once these limited compensatory mechanisms fail, the increased ICP can result in compression of the cerebral vasculature, causing further ischemia. Moreover, tissue shifts and herniation can occur, resulting in secondary brainstem dysfunction, terminally leading to respiratory or cardiac dysfunction. Following large hemispheric strokes in humans, ICP peaks at 2-5 days (Schwarz et al. 2001). ICP has not been investigated in mice following HI; however, breakdown of the BBB, subsequent vasogenic edema, and tissue shifts can be observed by 24 hours (Adhami et al. 2006). Thus, it is likely that mortality following HI in mice is similar to the condition noted in humans following large hemispheric stroke.

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\(^{12}\) The cytoplasm of all cells contains non-permeant anions (mostly proteins and phosphates); such an asymmetric distribution has been shown to lead to a predictable distribution of permeant ions across the cell membrane i.e., the Gibbs-Donnan Equilibrium, in which the total number of ions will be higher within the cell. The higher osmolarity of the cytosol would lead to the influx of water and hence cellular swelling. This does not occur under normal conditions due to active transport of ions that prevent such equilibrium from taking place.
Interestingly, my results indicate that seizures are significantly associated with mortality. The exact contribution of seizures to mortality following HI was not directly investigated in this study. It is tempting to speculate that seizures are a causal or worsening factor in both post-HI mortality and brain injury. A few experimental studies have explored this issue, particularly regarding early-onset, non-convulsive seizures in rats following middle cerebral artery occlusion (Williams et al. 2004; Williams et al 2006). In these studies, prophylactic treatments with anticonvulsive drugs collectively reduced the incidence of these non-convulsive seizures, mortality, and brain injury. In my experiments, post-HI prophylactic treatment with clinical dosages of diazepam and phenytoin significantly reduced seizure incidence and mortality. This result is in line with previous experimental studies, and further supports the hypothesis that early-onset seizures, whether convulsive or not, are critical for influencing overall post-ischemic outcome.

However, my observations indicate that when animals with brain injury are sorted according to the manifestation of seizures (or lack thereof), the magnitude of brain injury was not influenced by seizures. Moreover, the extent of ipsilateral brain injury was not significantly improved in animals treated with anticonvulsants. Fully interpreting these results is complicated by the association of seizures with mortality, as histological information in animals that died is lost. Consequently, only surviving animals were incorporated for analyses of brain injury. However, these results do suggest that, although seizures are associated with brain injury, seizures do not influence the extent of brain injury, at least with regards to the quantification methods chosen in this study. Furthermore, delaying treatment until seizure onset was ineffective in preventing mortality.

The lack of obvious neuroprotection combined with the failure of delayed anticonvulsant treatments in improving animal survival raises an interesting question: how does prophylactic anticonvulsant treatment suppress seizures and improve survival? One answer is that post-HI seizures may arise within (or spread to) the brainstem (see 10.6.2 below). This may in turn result in or accelerate the onset of terminal cardiac or respiratory dysfunction discussed above. Prophylactic treatment may reduce excitability in the brainstem and prevent the development (or spread) of seizures in the brainstem. Alternatively, prophylactic anticonvulsant treatments may lower core body temperature (Vartanian et al. 1996). Given that hyperthermia was observed in post-HI animals, and that fever has been correlated to worsened outcome following stroke.
(Adams et al. 2007) and also in the development of febrile seizures (Dube et al. 2009), and that hypothermia has shown promise in improving outcome following cerebral ischemia in humans and animal models (Adams et al. 2007), lowering body temperature may improve post-HI survival by preventing brainstem injury, dysfunction, or seizures.

Further investigations are required to fully examine these relationships. In particular, as delayed treatments only transiently suppressed seizures in my experiments, more efficient or chronic treatments may be necessary. It remains to be tested whether other anticonvulsants, particularly those with a wide pharmacological spectrum (Williams et al. 2004; Williams et al. 2006 Meierkord et al. 2010), are more effective in protecting against HI brain injury or in preventing seizures. Additionally, the potential effects of anticonvulsants on mitigating brainstem injury requires thorough examination (brainstem injury was not directly examined in this study). Finally, an investigation of the effects of hyperthermia on the development of post-HI seizures seems warranted.

To sum up, although there is a positive correlation between post-HI seizures, mortality, and brain injury, a clear causal relationship between seizures, mortality, and brain injury remains elusive. Nonetheless, the important point here is that that prophylactic treatment of post-HI seizures is effective in improving post-HI survival, and this result supports a reconsideration of the lack of prophylactic anticonvulsant therapy in humans following cerebral ischemia.

10.6 EEG activity during post-HI seizures

10.6.1 Non-convulsive EEG discharges

Previous studies have demonstrated that early-onset non-convulsive seizures occur following middle cerebral artery occlusion (MCAO) in rats, and that these non-convulsive seizures are associated with generalized electrographic spike-wave discharges (see Chapter 1, 1.5). Similarly, in my studies, ~22% of mice demonstrated non-convulsive EEG discharges during or shortly

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Moreover, in the opinion of this author, it is unclear as to how such a causal relationship could be conclusively determined in practice. Attempts to apply a reductionist approach to clearly and independently separate the mechanisms of ischemic brain injury, the mechanisms of post-ischemic seizure genesis, the contribution of seizures to ischemic brain injury, the contribution of ischemic brain injury to seizure genesis, the mechanisms of mortality, the contribution of ischemia to mortality, and the contribution of post-ischemic seizures to mortality would be an ambitious task.
following HI. However, there are several differences between these discharges and those reported following MCAO in rats:

1) HI-induced discharges were localized exclusively to ipsilateral brain regions, while the MCAO discharges tended to generalize bilaterally.

2) HI-induced discharges were on the order of seconds, while MCAO discharges lasted about a minute on average.

3) HI-induced discharges were dominated by faster frequencies ranging from 10-40Hz, while the MCAO discharges represented slower 1-4Hz spike-wave discharges.

4) The observed incidence of HI-induced discharges was lower (22% of mice) compared to MCAO discharges (85% of rats).

5) A maximum of two HI-induced discharges was observed in individual mice, while on average about a dozen discharges per post-MCAO rat were documented.

6) HI-induced discharges were mostly observed during HI and within a few minutes post-HI, while MCAO discharges were observed between 30 minutes and 3 hours post-MCAO.

Collectively, these observations suggest that although non-convulsive discharges occur during or shortly following HI, they likely arise through different cellular mechanisms than those reported following MCAO in rats. Thus, these discharges may represent a type of focal, non-convulsive epileptiform activity, resulting as a consequence of hypoxia-induced hyperexcitability. Further investigations are required to fully explore these issues.

10.6.2 Are post-HI convulsive seizures subcortical in origin?

In contrast to the non-convulsive HI-induced discharges described above and the non-convulsive seizures reported following MCAO in rats, a large proportion of post-HI mice developed multiple, recurrent, severe motor seizures within a few hours post-HI. Surprisingly, these convulsions were not observed to coincide with evident electrographic discharges from the ipsilateral or contralateral hippocampus or cerebral cortex. This result is controversial as seizures are ‘traditionally’ defined as aberrant, synchronized, excessive neuronal activity, which should
register in the EEG. There are several conjectures of the lack of observed electrographic discharges during post-HI motor seizures:

**Conjecture 1:** EEG discharges were not observed due to technical limitations.

This is unlikely due to technical limitations. This EEG recording method has demonstrated characteristic behavioral state-dependent EEG signals as described by others (*see Chapter 6, 6.1*). This indicates that the stated brain regions were effectively targeted. Furthermore, this exact recording method has permitted a reliable detection of EEG ictal discharges in juvenile mice following hypoxic challenges (Wais et al. 2009) and in adult rats following intra-hippocampal injections of cobalt (He et al. 2009). Furthermore, as described above, this recording method was successful in detecting non-convulsive discharges in ~22% of mice subjected to HI. Finally, EEG activity was extremely suppressed by HI. Thus, this EEG recording method is sound and is capable of detecting ictal discharges and ischemia-induced suppression of EEG activity.

**Conjecture 2:** EEG discharges were not observed due to movement artifacts.

Although, movement artifacts were a frequent problem when wired EEG recordings were carried out, no evidence of interictal activity or ictal discharges was observed prior to or following the severe motor seizures nor during relatively less-obscured milder convulsive activities. In addition, the telemetry recordings from the hippocampus and cortex are minimally affected by movement-related artifacts, and are thus optimal for detecting EEG discharges during convulsive seizures.

**Conjecture 3:** EEG discharges were not observed because recorded regions were severely injured.

EEG activity was strongly suppressed prior to seizures, and the regions recorded from demonstrated extensive brain injury. Thus, seizures may not arise in these structures as synapses and neurons were too damaged to support synchronized neural activity. However, if these seizures were truly generalized in nature, as the behavioral manifestations suggest, then EEG discharges would have been detected in the contralateral hemisphere, which was not injured and did not exhibit suppression in EEG activity. Further recordings from better preserved ipsilateral regions such as medial portions of the ipsilateral cerebral cortex are required to address this important issue; however, although plausible, I consider this interpretation to be dubious.
Conjecture 4: EEG discharges were not observed because seizures occur in the brainstem or spinal cord.

The clear generalized manifestations of post-HI motor seizures implicate one of the three hierarchical levels of motor control. These are the forebrain, brainstem, and spinal cord. These levels can further be modulated by input from the cerebellum and basal ganglia. As EEG discharges were not observed in the forebrain during post-HI motor seizures, my results implicate either the brainstem or spinal cord in generating the post-HI convulsions. In line with this hypothesis, Cohn (1979) demonstrated that bilateral occlusion of the common carotid artery in gerbils resulted in motor seizures, and that these seizures were not associated with electrographic discharges in epidural cortical EEG recordings. Interestingly, Cohn (1979) detected discharges in recordings from the thoracic spinal cord. In conjunction with Cohn (1979), I suggest that post-ischemic generalized motor seizures can originate either in the brainstem or spinal cord in the absence of forebrain involvement.

Convulsive seizures in rats have been well-studied following the administration of pro-convulsant drugs or following focal electrical stimulation (Gale 1992; Velíšek 2006; Velíšková 2006). In general, convulsive seizures triggered in this manner propagate along specific anatomical pathways; and the specific pattern of propagation, the pathways utilized, and brain regions involved will influence the behavioral manifestations.

The absence of clear EEG discharges in the ipsilateral and contralateral cortex and hippocampus during post-HI convulsive seizures suggests that these regions are not recruited during the motor seizures. In particular, the absence of any detectable hippocampal involvement strongly suggests that post-HI seizures are not limbic in origin: in seizures of limbic origin, the hippocampus is usually one of the first regions to exhibit ictal discharges (Gale 1992). Further, the absence of ictal discharges in contralateral cortical or hippocampal areas indicates that these seizures do not generalize to bilateral forebrain regions. Finally, limbic motor seizures are typically characterized by bilateral forelimb clonus, facial and mouth clonus, with rearing and falling (Gale 1992; Velíšková 2006). These behavioral manifestations were not observed in post-HI mice.

The motor convolution most commonly observed in post-HI mice is similar to the so-called ‘explosive running-bouncing clonic seizure’ (Gale 1992). This type of motor activity has been
frequently observed in rodents following the systemic administration of pro-convulsant drugs such as pentylenetetrazol, picrotoxin, and bicuculine (Gale 1992), in spontaneous or sensory-evoked seizures in the genetic epilepsy-prone rat (GEPR) (Gale 1992; Jobe and Browning 2006), in alcohol-withdrawal seizures (N’Gouemo and Rogwaski 2006), and hypoglycemic seizures (Velíšek et al. 2008). Typically, the ‘explosive running-bouncing clonic seizure’ begins with clonic movements of all four limbs resulting in a sudden, explosive, running or jumping fit, and results in a loss of posture, with clonic movements of the limbs continuing while the animal is on its side or back. I consistently observed these behavioral correlates during post-HI motor seizures. In addition to these clonic seizures, post-HI mice usually exhibited tonic extensions of the forelimb, and these usually occurred at the end of the seizure. This is a typical manner for seizures to end in animals following maximum convulsive stimulation with pro-convulsant drugs such as pentylenetetrazol (Gale 1992).

The brainstem is believed to be critically involved in the genesis of these motor manifestaions, and, importantly, the motor manifestations of these seizures do not require the integrity of the forebrain (Gale 1992; Jobe and Browning 2006; N’Gouemo and Rogwaski 2006). A number of midbrain sites have been implicated in the genesis of these seizures, including the superior colliculus, inferior colliculus, substantia nigra, periaqueductal gray, and the midbrain reticular formation. A brainstem site critically involved in the audiogenic explosive running-bouncing clonic seizures is the inferior colliculus, a midbrain nucleus which is normally part of the auditory sensory pathways. In line with this view, it has been demonstrated that rats subjected to transient global cerebral ischemia (induced by transient chest compression) exhibited a reduced threshold to audiogenic seizures (Iyer et al. 1995; Reid et al. 1996). It would be interesting to determine whether post-HI seizures can be triggered by sensory stimuli such as sound.

Collectively, these observations suggest post-HI seizures may arise directly within the brainstem and/or spinal cord and result in motor manifestations without forebrain recruitment.

However, the obvious question, then, is how these seizures are triggered in the brainstem or spinal cord following HI in adult mice. The brainstem derives most of its arterial blood from branches of the vertebral and basilar arteries (Tatu et al. 2009). Thus, as blood supply in these regions does not directly fall under the territory of the internal carotid artery, it is unclear why they would be involved in post-HI seizures. However, large regions of the midbrain are supplied
by the posterior circle of Willis, at least in humans. For example, perforating branches of the posterior cerebral and posterior communicating arteries form a plexus referred to as the interpeduncular fossa/arteries, and these supply large regions of the midbrain; furthermore, the collicular artery that supplies the inferior colliculus is a branch of the posterior cerebral artery (Tatu et al. 2009). As discussed above, brainstem regions such as the inferior colliculus and reticular formation are critically involved in convulsive motor seizures in rats. The arterial supply to midbrain regions from the posterior circle of Willis suggests that the midbrain may be ischemic during HI (See 10.2: ‘How does this result in panhemispheric infarction…’). Midbrain injury was not directly examined in this study, and future studies are required to fully elucidate the structural damage and involvement of these regions (see 11.3.3 below).

There is an alternative viewpoint. It has been stressed that specific brain regions can trigger a seizure in a distinct but anatomically connected region, without itself being primarily involved in the generation of ictal EEG discharges (Gale 1992). Under this viewpoint, the lack of observed discharges during motor seizures in post-HI mice does not necessarily mean these regions are not involved in triggering seizures in the brainstem. Via anatomical interconnections, multiple brain regions may modulate neuronal activity in the brainstem.

In the absence of further experimentation, further discussion of these issues will remain speculative. A resolution of these issues requires that recordings from brainstem regions be initiated (see 11.3.3 below). At any rate, seizures and/or secondary damage in regions such as the brainstem may be a contributing factor to post-HI mortality as this region is involved in regulating critical involuntary functions such as cardiovascular and respiratory functions.
11 Conclusions, novelty, and outlook

Here, I would like to summarize the main conclusions and novelties of this study and then discuss future experiments.

11.1 Main conclusions

1. HI results in ipsilateral brain injury in a large proportion of mice. Brain injury occurs across large regions of the ipsilateral hemisphere, and is likely caused by a profound decrease in ipsilateral cerebral blood flow. Brain injury in these animals is correlated to a severe suppression of ipsilateral EEG activity, disruptions of intrinsic brain rhythms, and synaptic dysfunction in vitro.

2. Some mice appear to be resistant to HI-induced brain injury. Compared to the mice described in point 1 above, these resistant mice do not exhibit large and conspicuous brain lesions when assessed by gross histological inspection. Moreover, they experience a relatively milder reduction in cerebral blood flow, and a relatively milder and reversible alteration in EEG activities, as well as no major synaptic defects assessed in vitro. However, specific markers of cellular degeneration revealed that these animals exhibit subtle cellular degeneration that may escape standard histological assessments.

3. EEG activity is a good marker for assessing synaptic dysfunction during and following HI. And EEG activity may be a good experimental marker for distinguishing animals described in points 1 and 2 above. Thus, the EEG may be a useful technique for reducing experimental variability and may have clinical value in assessing outcome following stroke.

4. HI results in severe early-onset generalized motor seizures in ~44% of mice. This agrees with the observation of post-stroke generalized motor seizures in humans. These seizures are significantly associated with the severe brain injury described in point 1 above. Seizures are also
significantly associated with increased mortality. This suggests that post-HI motor seizures are intimately associated with the process of ischemic brain injury and that they may contribute to post-HI mortality. Further, these observations agree with human epidemiological studies that have found an association between post-stroke seizures, severe brain injury, and mortality. Thus, post-HI motor seizures may be a major pathological component of the HI model in adult mice. It therefore follows that HI is a useful experimental tool to model post-stroke convulsive seizures in a controlled laboratory setting.

5. Prophylactic treatment with anticonvulsants reduces seizure incidence and mortality but does not influence the extent of brain injury as described in point 1 above. Delaying treatment with anticonvulsants until seizures are observed does not reduce mortality. This suggests that prophylactically treating seizures improves post-HI survival.

6. Non-convulsive EEG discharges can occur in a portion (~22%) of mice during and shortly following HI. Animals were completely immobile prior to and following these discharges. These discharges were not associated with overt behavioral manifestations such as motor convulsions. The observation of non-convulsive discharges agrees with clinical observations of non-convulsive post-stroke seizures in humans and also with previous observations of non-convulsive seizures following experimental cerebral ischemia in rats.

7. Curiously, no classical electrographic discharges were observed to coincide with the motor seizures described in point 4 above, neither in recordings from the ipsilateral or contralateral hippocampus and cerebral cortex. This implicates deep subcortical structures such as the brainstem in the genesis of post-HI seizures.

11.2 Novelty

1. First electrophysiologically characterized synaptic dysfunction following hypoxia-ischemia in adult mice. Further, this is one of the most detailed long-term assessments of the effects of experimental cerebral ischemia on EEG activity, and the most detailed correlation of EEG activity to outcome following experimental cerebral ischemia.

2. First attempt to understand why some animals are resistant to HI. In the context of HI, this is the first demonstration of an association of variable brain injury and mortality to variable cerebral blood flow and EEG alterations. Moreover, it is the first description of subtle brain
injury as a consequence of HI in a subset of mice. Thus, HI may lead to a complex spectrum of brain injuries post-HI.

3. First report of early-onset motor seizures following HI. To my knowledge, this is also the first detailed characterization of early-onset convulsive seizures following cerebral ischemia in adult rodents.


5. First demonstration of the benefits of anticonvulsants in the context of convulsive seizures following focal cerebral ischemia in adult rodents.

6. First demonstration of non-convulsive discharges during HI in adult mice.

7. First demonstration that early-onset post-ischemic seizures are not associated with electrographic discharges in intracranial EEG recordings from the hippocampus or cerebral cortex following ischemia in mice.

11.3 Future experiments

11.3.1 Resolving the mechanisms of post-HI brain injury

As discussed above, HI results in cerebral ischemia/oligemia. However, the mechanisms for the hypoxia-induced declines in cerebral blood flow (CBF) are not understood. I proposed three possible mechanisms above (see Chapter 10, 10.2).

1. Mean arterial blood pressure

As discussed above (see Chapter 10, 10.2), reduced mean arterial blood pressure (MAP) during systemic hypoxia may result in severe declines in ipsilateral cerebral blood flow in the HI model. Mean arterial and left ventricular pressure can be examined in non-anesthetized mice via commercially available implantable telemetry-based transmitters. For example, the PhysioTel® PA series transmitters available from Data Sciences Interantional (DSI) employ pressure transmission catheters and are suitable for telemetry recordings in free-moving mice.
2. Intravascular coagulation:

As I argued above (see Chapter 10, 10.2), I do not think that intravascular coagulation can explain the declines in CBF during HI. Nonetheless, I propose the following experiment to test the hypothesis that hypoxia-induced intravascular coagulation results in cerebral ischemia. The experiment requires animals to be anesthetized as described during the laser Doppler experiments above (see Chapter 5, 5.1). The right common carotid artery (RCCA) should be ligated, and this should be followed by respiratory hypoxia (8% O₂). The experimenter should monitor cerebral blood flow carefully. Once CBF is reduced to 85% of baseline the experimenter should attempt the following:

a) Test the effects of immediate return to normoxic air flow.

b) Test the effects of multiple anticoagulant/thrombolytic agents, such as heparin or rtPA. The drugs can be administered systemically through the tail vein or through the contralateral carotid artery.

If hypoxia-induced intravascular coagulation is responsible for the secondary ischemia/oligemia induced by hypoxia, then these measures should reverse the decline in cerebral blood flow.

These experiments should be repeated at the end of the hypoxic episode to confirm the observation of reperfusion deficits reported by Adhami et al (2006).

2. Disruption of vascular autoregulation:

To test the hypothesis that low tissue oxygen tension is responsible for secondary vasoconstriction of the cerebral vasculature (see Chapter 10, 10.2), I recommend the following experimental paradigm. Again, as described above, HI should be carried out in anesthetized animals. I recommend adapting the approaches of Shin et al (2006) and Takano et al (2007).

To examine the spatiotemporal characteristics of cerebral blood flow (CBF), I recommend the use of laser speckle flowmetry or laser-Doppler based approaches, which can be carried out through the intact, unexposed skull. A small hole can be made in the skull through which a recording electrode can be placed to examine the DC shift associated with spreading depression. A second hole can be drilled nearby to place an oxygen-sensitive microelectrode. The objective
of this experiment will be to determine a) the dynamics of tissue oxygen levels during HI b) whether cortical spreading depression does indeed occur during HI, and c) to determine whether the onset of spreading depression is associated with secondary reductions in CBF.

To examine the possibility of vasoconstriction induced by HI, I recommend the following experiment. A closed cranial window should be surgically implanted to directly visualize the pial arterioles for vessel diameter measurements during cortical spreading depression. Images can be acquired via reflection of visible light directly via a CCD camera. Alternatively, arterial blood can be loaded with a fluorescent indicator such as FITC-dextran via injection through the tail vein, and images of pial vessels acquired with a two-photon microscope. Incidentally, NAD(P)H/NAD(P) redox status measurements may also be useful, and can be examined by taking advantage of the auto-fluorescent characteristics of this metabolite.

11.3.2 Explaining the variability in post-HI outcome

I proposed one major explanation for the variability in post-HI brain injury: intra-strain variability in the cerebral vasculature (see Chapter 10, 10.2).

To provide evidence for the hypothesis that intra-strain variability in the cerebral vasculature is responsible for the variability in post-HI brain injury, the following preliminary experiment is recommended. Animals should be subjected to HI. At 72 hours post-HI, the cerebral vasculature should be labeled by intra-cardiac perfusion of dyes such as India ink or carbon latex. The brain can be removed and the arterial circle of Willis can be examined for abnormalities in the component arteries. This can then be correlated to the extent of brain injury in the same animals.

Alternatively, if funds and equipment are available, then blood flow through the circle of Willis can be examined in vivo in anesthetized animals with magnetic resonance imaging angiography (Beckmann 2000), and this could then be correlated to outcome.

Alternatively, the duration of hypoxia can be increased or the partial pressure of oxygen in the inspired gas can be reduced from 8% to 6% (my preliminary results have indicated that 4% O₂ is fatal). These efforts may reduce variability.
11.3.3 Determining the source of post-HI motor seizures

In section 10.6.2, I drew parallels between the behavioural manifestations of post-HI motor seizures to brainstem motor seizures observed in rodents following the systemic administration of pro-convulsant drugs such as pentylenetetrazol, picrotoxin, and bicuculine, in spontaneous or sensory-evoked seizures in the genetic epilepsy-prone rat (GEPR), in alcohol-withdrawal seizures, and hypoglycemic seizures. I therefore hypothesized that post-HI motor seizures may arise from deep subcortical structures such as the brainstem or spinal cord. Thus, EEG recordings should be carried out from these regions. However, multiple brainstem sites have been implicated in the genesis of brainstem motor seizures, including the superior colliculus, inferior colliculus, substantia nigra, periaqueductal gray, and the midbrain reticular formation. Thus, resolving the sites of genesis, spread, and recruitment is technically challenging, and will require extensive EEG recordings, ideally with multiple recording electrodes.

Alternatively, clues to the location of seizure genesis sites may be provided by techniques other than the EEG. Functional magnetic resonance imaging utilizing the blood-oxygen-level dependence (BOLD) signal may correlate oxygen utilization to aberrant neural activity associated with seizures. An alternative approach would be to examine c-Fos gene expression in different brain regions: this gene is upregulated during increased synaptic activity and may indicate seizure activity. Additionally, the uptake $^{14}$C-2-deoxyglucose may indicate an increase in glucose uptake during a seizure.

If specific brainstem sites or the spinal cord is found to be the site of seizure genesis, it will next be necessary to begin determining the mechanisms of seizure genesis. This could be examined in a number of ways. I would first recommend the insertion of cannulas to allow for the infusion of pharmacological agents. For instance, it would be interesting to note whether infusing agents that enhance excitability and are implicated in excitotoxicity, such as glutamate, into the midbrain can trigger seizures in control animals that behaviorally resemble post-HI seizures. Secondly, drugs that block synaptic transmission such as glutamate receptor antagonists should be tested to determine whether they can inhibit post-HI seizures. Additionally, the development of an in vitro acute midbrain or spinal cord slice preparation may be useful in the general investigation the cellular mechanisms of seizure genesis.
This concludes the main body of this thesis. I have included appendices that contain supplementary data and reference materials that support and augment the results. However, my main arguments have been presented.
References


Appendix I. Cerebral collateral flow: a review of the clinical and experimental impact

Introduction

The extent of collateral blood flow to cerebral territories following a focal obstruction of arterial supply to the brain is affected by anatomical variability in the cerebral vasculature, and this in turn influences the risk and outcome of ischemic stroke in humans. Interestingly, rodents also exhibit a number of anatomical variations that may influence the extent of cerebral collateral flow during experimental cerebral ischemia; however, multiple inter- and intra-species anatomical differences exist and can lead to variability in outcome in several models of experimental cerebral ischemia. Yet, to date, a comprehensive description and an in depth understanding of the effects of collateral flow in rodents is lacking. Here, I review the similarities, differences, and variations in anatomical pathways of cerebral collateral blood flow in humans and rodents.

Abbreviations

ACA, anterior cerebral artery; AcomA, anterior communicating artery; AChA, anterior choroidal artery; BA, basilar artery; FTP, fetal-type posterior cerebral artery; ICA, internal carotid artery; MCA, middle cerebral artery; mPcomA, mouse posterior communicating artery; PCA, posterior cerebral artery; PcomA, posterior communicating artery; SCA, superior cerebellar artery.

The circle of Willis in humans

The arterial supply to the human brain has been extensively reviewed elsewhere (Tatu et al. 2001; Scremin 2004a; Kumaral et al. 2009; Tatu et al. 2009). Here, I will provide an overview of the pertinent features relevant to this exposition.

Four arteries ascend to supply blood to the brain; the right and left internal carotid arteries (ICA) and the right and left vertebral arteries. The latter two unite to form the basilar artery (BA). The BA and the two ICAs form an interconnected anastomotic arterial circle (the circle of Willis) at
the base of the brain. The circle of Willis, then, is the hub of arterial blood to the cerebral hemispheres (*Figure 1*).

The circle of Willis can be broadly divided into anterior and posterior regions. The anterior circulation derives from the common carotid artery (CCA), which branches into an external (ECA) and internal carotid artery (ICA). The ICA courses towards the circle of Willis, where it gives off its terminal or cerebral branches, which include the middle cerebral artery (MCA), the anterior cerebral artery (ACA), the relatively thinner posterior communicating artery (PcomA), and, usually, the anterior choroidal artery (AChA). The main continuing branch is the MCA, while the smaller ACA forms the anterior portion of the circle of Willis. The anterior communicating artery (AcomA) forms an anastomosis with the opposite ACA, thus linking the arterial supply of both ICAs. This, then, completes the anterior half of the circle of Willis (*Figure 1*).

The posterior circulation of the cerebral hemispheres arises from the vertebrobasilar system, which is also the source of the blood supply to the brainstem and cerebellum (not discussed here). The vertebrobasilar system derives from the left and right vertebral arteries, which are branches of the subclavian arteries. At the lower border of the pons, the vertebral arteries merge to form the basilar artery (BA). The BA terminates as a bifurcation into two posterior cerebral arteries (PCA). Of relevance to this discussion, two superior cerebellar arteries (SCA) arise within or just caudal to the rostral division of the basilar into the two PCAs. The initial portion of the PCA receives a posterior communicating artery (PcomA) from the ICA. The region prior to receiving the PcomA is sometimes referred to as the mesencephalic artery, precommunal portion, or P1 segment of the PCA. The anastomosis with the PcomA completes the posterior half of the circle of Willis, thus connecting the ICAs with the vertebrobasilar system (*Figure 1*).
Appendix I. Figure 1. The arterial circle of Willis in humans and rats

The circle of Willis is an anastomotic arterial circle at the base of the brain that connects the internal carotid arteries with the vertebrobasilar circulation. Left, illustration of the typical human circle of Willis. Of relevance to this discussion, note that in humans the posterior communicating artery is a relatively thin artery that connects the internal carotid artery with the posterior cerebral artery. Right, illustration of the typical rat circle of Willis. Note that in rats the posterior communicating artery is relatively larger and is of a size and caliber comparable to the middle and anterior cerebral arteries. There has been contradictory usage of the terminology of the posterior communicating artery in rodents. The terminology shown in this figure is the classical nomenclature. However, in experimental studies of cerebral ischemia several authors refer to the initial segment of the posterior cerebral artery that forms an anastomosis with the basilar artery as the posterior communicating artery. And this vessel is frequently reported as being absent or hypoplastic, particularly in C57BL/6 mice. Figure reproduced from Lee et al. (1995).
Arterial territories of the cerebral hemispheres in humans

The arterial circulation of the cerebral hemispheres is classically divided into leptomeningeal arteries and perforating arteries (Scremin 2004a; Tatu et al. 2009). The leptomeningeal arteries are terminal branches of the MCA, ACA, and PCA that form an interconnected anastomotic network on the surface of the cerebral hemisphere, and they give rise to branches that penetrate the cerebral cortex and underlying white matter. For this reason these arteries are also referred to as pial or superficial arteries. The size and scope of the areas supplied by the leptomeningeal branches varies across the dorsal-ventral axis of the cerebral hemisphere; and a detailed description of territories supplied by these arteries is beyond the scope of this brief exposition. A few general comments on some of the cortical branches relevant to this discussion are nonetheless warranted. At its greatest, the territory supplied by the cortical branches of the ACA contains most of the medial surface of the cerebral cortex; at its smallest, the territory can be restricted to the anterior part of the frontal lobe. The cortical branches of the MCA supply most of the temporal lobe, anterior-lateral frontal lobe, and parietal lobe; at its greatest, the MCA can supply almost the whole lateral surface of the hemisphere. The PCA produces branches that supply the inferior-medial portions of the occipital and temporal lobes. Notably, a branch of the PCA, the longitudinal hippocampal artery, also supplies the hippocampus. In addition, perforating arteries arise from the circle of Willis or its immediate branches and penetrate the brain directly. The arterial territories of the perforating branches are extensive and too detailed to be discussed here. However, they encompass broad regions such as the hypothalamus, thalamus, basal ganglia, and midbrain.

Collateral circulation in humans

Collateral circulation can be defined as blood flow established through indirect branches when the supply through the main vessel is obstructed. Collateral circulation in the brain is important for maintaining a sufficient level of cerebral blood flow during ischemic stroke. Depending on where the obstruction takes place, a number of routes of collateral flow to the cerebral hemispheres are available. Of particular relevance to this discussion, four main systems are of interest (Gillilan 1974; Lee 1995; Kumaral et al. 2001; Scremin 2004a):

a) Collateral flow can originate from the ICA, and can supply the contralateral hemisphere via the anterior circle of Willis. In this route, blood from the ICA passes through the ACA and then
through the AcomA. From here blood can flow along the cortical branches of the contralateral ACA anterogradely; additionally, blood can pass retrogradely to supply the contralateral MCA, and then anterogradely along its branches.

b) Collateral flow can originate from the vertebrobasilar system. From the BA via the PCA, blood can enter into the PcomA and then anterogradely supply the MCA and ACA.

c) Another collateral pathway is between the leptomeningeal branches of the cerebral arteries, which form a complex anastomotic network on the surface of the cerebral hemispheres. They may result in an important connection between the ICAs and the vertebrobasilar system. The frequency and size of anastomoses is highest between the terminal branches of the anterior and middle cerebral arteries, followed by those between the middle and posterior cerebral arteries, with the lowest numbers between the anterior and posterior cerebral arteries.

d) A final major collateral pathway is extracranial-to-intracranial through the orbit (the cavity or socket of the skull in which the eye and its appendages are situated). The ophthalmic artery is a branch of the cavernous portion of the ICA. The facial branches of the external carotid artery anastomose extensively with the ophthalmic artery; thus, blood flow can originate from an extracranial route, and, in a retrograde fashion, flow through the ophthalmic artery into the ICA and then the circle of Willis.

Additional notable sources of collateral flow include an anastomotic network that covers the superior and inferior colliculi, formed by branches of the PCA and SCA; arterial connections between the meningeal arteries of the dura mater; and anastomoses within the vertebrobasilar system.

**Anatomical variations in the circle of Willis in Man**

Anatomical variations in the circle of Willis have been described in several species, including humans, cows, sheep, goats, mice, rats, gerbils, and pigs (Lee 1995; Scremin 2004a; Scremin 2004b; Ashwini et al. 2008; Howells et al. 2010). It has been estimated that only 20% of human individuals have a ‘typical’ circle of Willis as described above; and these anatomical variations are associated with an increased risk of stroke and more extensive brain injury and poorer outcome following ischemic stroke and carotid artery stenosis (Battacharji et al. 1967; Schomer et al. 1994; Hartkamp et al. 1999; Chaves and Caplan 2001; Sawada et al. 2001; Schwarz et al.
The anterior circle of Willis is variable among normal people (van der Zwan et al. 1992; Lee 1995; Sawada et al. 2001; Scremin 2004a; Jaramillo et al. 2006; Kapoor et al. 2009; Manninen et al. 2009; Howells et al. 2010). The proximal ACA may be completely absent in up to 2% of people. Typically, the proximal ACA is about 2.6 mm in diameter, yet it may be hypoplastic (narrow and string-like with a diameter less than 1 mm) in up to 11% of people. More importantly, the AcomA can be completely absent in up to 7% of normal people. The typical diameter of the AcomA is 1.4 mm with a total length of 3.3 mm, and it may be hypoplastic in up to 37% of people. Interestingly, this tiny artery may exist as duplicates and triplicates in some people.

The posterior circle of Willis is highly variable in normal people (Battacharji et al. 1967; van der Zwan et al. 1992; Schomer et al. 1994; Lee 1995; Chaves and Caplan 2001; Scremin 2004a; van Raamt et al. 2006; Chuang et al. 2008; Manninen et al. 2009; Howells et al. 2010). Variations in the size of the PCA have been described; for instance, one of the PCAs can be unusually small in 24% of people or unusually large in 29% of individuals (Chaves and Caplan 2001). Another important variation of the PCA is referred to as the fetal-type PCA (FTP) (Scremin 2004a; van Raamt et al. 2006). In early embryonic development, the PCA develops as a continuation of the PcomA, and, at this stage, it is therefore technically a branch of the ICA. In later fetal stages of development, the PCA begins to receive its blood from the vertebrobasilar circulation, and, at this point, it is then formally considered as a terminal branch of the BA; however, in up to 29% of people, a variation of this developmental scheme occurs, and the connections to the vertebrobasilar circulation may not form properly (Scremin, 2004a; van Raamt et al. 2006). This can range from partial hypoplastic connections through the P1 segments of the PCA to a complete absence of any connection to the vertebrobasilar system; and this can occur unilaterally or bilaterally. These anatomical variations are respectively referred to as a partial or full, unilateral or bilateral FTP. In these cases, blood supply to the PCA is either mostly or solely derived from the ICA. Moreover, under these configurations, the leptomeningeal collateral connections to the vertebrobasilar system will either be compromised or precluded from forming, as the PCA, MCA, and ACA will all derive from the ICA. In the event, then, of a full, bilateral
FTP, the anterior circulation to the cerebral hemispheres is effectively cut-off from the posterior circulation.

Additionally, the patency and origin of the PcomA has also been reported to be variable across individuals. It has been reported as hypoplastic or absent in one or both sides in up to 38% of people (Battacharji et al. 1967; van der Zwan et al. 1992; Schomer et al. 1994; Lee 1995; Chaves and Caplan 2001).

**Cerebral vasculature and collateral flow pathways in rats, mice, and gerbils**

**Rat**

The cerebral vasculature of the Rat has been extensively well-characterized and is generally similar to that in humans (Greene 1955; Moffat 1961a; Moffat 1961b; Zeman et al. 1963; Brown 1966; Coyle 1975; Coyle 1976; Coyle 1978; Coyle and Jokelainen 1982; Lee 1995; Sbarbati et al. 1996; Reese et al. 1999; Scremin 2004b). Here I will provide a brief overview of the rat arterial circle of Willis based on the previously mentioned reports.

As in humans, two common carotid and two vertebral arteries supply the cerebrum, brain stem, cerebellum, and cervical spinal cord. Notably, however, there are a number of important differences between circle of Willis in rats and humans (Lee 1995; Scremin 2004b). In rats the AcomA is generally absent and is present only in ~20% of animals; instead the two ACAs usually fuse to form a single azygos ACA. Prior to the fusion, the ACA also gives off a large olfactory artery. A similar vessel to the rat olfactory artery exists transiently in humans during embryonic development. This presumably reflects the relatively greater reliance on olfaction in rodents.

Differences in the posterior circle of Willis have also been well-documented (Lee 1995; Scremin 2004b). However, a brief important note on nomenclature is warranted prior to an exposition of posterior circle of Willis in rodents. There has been certain amount of confusion and contradictory usage regarding the terminology of the PCA and PcomA in rats, as the branching patterns and relative sizes of these two arteries are different in rats than in humans. As discussed above, in humans, the PcomA is a relatively thin branch of the ICA that connects the PCA with the ICA; and the patency of this artery is frequently compromised. However, in rats, the PcomA is relatively larger than the PCA, and is of a size and caliber comparable to the rat MCA and
ACA (Figure 1). Despite these differences, some authors have decided to maintain the same terminology for the PcomA and PCA as is universally used in humans, and these arguments are supported by ontogenetic evidence (Lee 1995; Scremin 2004b). However, from a functional perspective, there is an alternative viewpoint. Because of the relative size of the PcomA in rats, the posterior circulation of the cerebral hemispheres may be derived mainly from the ICA. Under this scheme, the terms PcomA and PCA should arguably be reversed. It seems that this reversed terminology is currently predominating in the literature of experimental cerebral ischemia in rodents, and it is important to keep this in mind (see below discussion of this point in mice). However, because it is generally believed that the patency of the posterior circle of Willis in rats is relatively well-preserved, the terminology does not affect the basic geometry of the circle of Willis and the pathways of collateral flow from the vertebrobasilar system. Putting aside semantics for a moment, the main point here is that the posterior supply to the cerebral hemispheres in rats may experience a relatively greater contribution from the ICA; it should further be noted, then, that the situation in rats is arguably similar to what is observed in a partial FTP in humans described above.

In rats, it is common for the BA to terminate as a bifurcation into two superior cerebellar arteries (SCA), and a PCA arises from each SCA at a variable distance from this bifurcation (Scremin 2004b). Alternatively, as commonly described in humans, the PCA can arise directly from the BA. An extensive amount of variability can occur even within rat strains, and the situation described above is rarely symmetric; for example, it is not uncommon to observe both PCAs to arise unilaterally from the same SCA, with one PCA then crossing over to the other side. Interestingly, the PCA usually forms an anastomosis with its contralateral counterpart. Finally, the relative contribution of the PCA to the size of circle of Willis in rats is greater than in humans, and it has been suggested that this may relate to the greater relative size of the rat hippocampus, which derives its main arterial supply from the PCA via the longitudinal hippocampal artery (Coyle 1975; Coyle 1976; Coyle 1978; Scremin 2004b).

Moving away from the circle of Willis for a moment, as discussed above, extracranial to intracranial collateral flow to the ICA via the ophthalmic artery may be important in humans. In rats, however, the major blood supply to the orbit is via the external ophthalmic artery, which is a terminal branch of an interesting vessel known as the pterygopalatine artery (also known as the stapedial artery; Scremin 2004b). This vessel deserves some further discussion. This artery is a
branch of the ICA prior to its entering the cranium, and it is variably present across species; for example, it can range from a rudimentary vessel in humans to a fully developed artery comparable in size to the ICA in rats. This artery courses intracranially, and it is frequently observed in magnetic resonance angiography of the circle of Willis in rodents (e.g. Beckmann et al. 1999; Beckman 2000). During its intracranial course, it gives off a single branch, the middle meningeal artery, which supplies the cerebral dura matter. The artery emerges from the cranium at the petrotympanic fissure and gives rise to a number of branches that supply extracranial structures, consequently resulting in a number of extracranial to intracranial anastomotic circles that do not occur in humans (Scremin 2004b). It therefore appears that, in rats, the pterygopalatine artery may be an important source of collateral flow.

Further, as in humans, an extensive anastomotic network of the leptomeningial arteries exists in rats (Lee 1995; Scremin 2004b). The number of collaterals between the anterior and middle cerebral arteries is 4-5 times higher in rats than humans, averaging at about 29 connections per hemisphere with a diameter of ~120μm and a distribution higher towards the midline (Coyle and Jokelainen 1982). Of note, a large branch from the middle cerebral artery, the rhinal artery, running almost horizontally in the caudal direction through the rhinal fissure, receives anastomoses from the terminal branches of the MCA and usually joins branches of the PCA.

In summary, the anatomy of the circle of Willis and collateral flow pathways are similar in rats and humans. The main difference with regards to humans is the greater size of the PcomA relative to the PCA in rats, and this has led to some confusion regarding the nomenclature of this important artery. Further, there do not appear to be many reports on variability in the intactness of the circle of Willis within or across rat strains: in particular, several studies have indicated that the circle of Willis is relatively well-formed in rats (Brown 1966; Lee 1995; Ozdemir et al. 1999; Scremin 2004b; Howells et al. 2010), which is in contrast to humans and, as we shall see below, also to mice and gerbils. However, a number of inter-strain or inter-vendor related differences in the caliber of the PcomA, branching patterns of the MCA, kinking of the ICA, and the pial anastomotic network have been reported, and these anomalies may at least partially explain some of the variability in ischemic brain injury across several rat strains following experimental cerebral ischemia (Yamori et al. 1976; Rieke et al. 1981; Duverger et al. 1988; Fox et al. 1993; Iwasaki et al. 1995; Herz et al. 1996; Oliff et al. 1997; Ozdemir et al. 1999; Dittmar et al. 2006;
Marosi et al. 2006; Durukan and Tatlisumak 2007; Kim et al. 2008; Durukan and Tatlisumak 2009; Howells et al. 2010).

**Mice**

Mice are increasingly being employed in studies of experimental cerebral ischemia due to the popularity of genetically engineered animals. Several inbred, outbred, and hybrid mouse strains are currently employed. In comparison to rats, however, a relatively detailed assessment of the cerebral vasculature is lacking, and this further confounded by the plethora of mouse strains utilized in experimental studies. However, in mice, the reports of vascular anomalies and their association with variability in outcome following experimental cerebral ischemia is far better documented than in rats. Several studies have investigated the cerebral vasculature in mice (Barone et al. 1993; Connolly et al. 1996; Fujii et al. 1997; Yang et al. 1997; Kitagawa et al. 1998; Maeda et al. 1998; Murakami et al. 1998; Beckmann et al. 1999; Ozdemir et al. 1999; Beckmann 2000; Majid et al. 2000; Wellons et al. 2000; Kelly et al. 2001; Tsuchiya et al 2003; Furuya et al. 2004; Krucker et al. 2004; McColl et al. 2004; Okuyama et al. 2004; Yonekura et al. 2004; Tamaki et al. 2006; Cho et al. 2007; Dorr et al. 2007; Zhen and Doré 2007; Arboleda-Velasquez et al. 2008; Todo et al. 2008; Meng et al. 2009). Below I shall discuss some of the anatomical variations documented in the above studies that are relevant to this exposition.

The basic architecture of the circle of Willis in mice is similar to that in humans and rats. Similar to humans, and in contrast to rats, the majority of the above mentioned studies have indicated that a clear AcomA seems to be present in mice; moreover, to our knowledge, there have been no reports documenting the absence or hypoplasticity of this vessel. However, similar to rats, an azygos ACA has been reported in CBA mice (Dorr et al. 2007). Importantly, as discussed above in rats, there seems to be some contradictions regarding the nomenclature of the PCA and PcomA. In general, the terminology of the PCA and PcomA employed in most of the above reports, including the commonly used C57BL/6 strain, is in contrast to what is classically adopted in rats and humans (*but see* Dorr et al. 2007). The vessel corresponding to the PcomA in humans and classically in rats is usually referred to as the PCA in mice, while the vessel corresponding to the P1 segment of the PCA in humans and classically in rats is referred to as the PcomA in mice (see above discussion of this confusing point in rats).
I will adopt this terminology for the remainder of this discussion, and will refer to this vessel as the mouse (m)PcomA. The mPcomA is a relatively shorter vessel than the PCA, is usually described as thinner, and this anastomosis may be located relatively more proximally than in rats. Importantly, the mPcomA has frequently been reported or suspected by a number of investigators as being either absent or hypoplastic, either unilaterally or bilaterally, both within and across mice strains (Barone et al. 1993; Fujii et al. 1997; Yang et al. 1997; Kitagawa et al. 1998; Murakami et al. 1998; Beckmann et al. 1999; Ozdemir et al. 1999; Beckmann 2000; Wellons et al. 2000; Kelly et al. 2001; Krucker et al. 2004; McColl et al. 2004; Yonekura et al. 2004; Tamaki et al. 2006; Cho et al. 2007; Zhen and Doré 2007). This abnormality appears to be strain dependent; the posterior circulation has been reported or suspected to be impaired in C57BL/6, BALB/c, CFW, CD1, ddy, 129X1/SvJ, and Swiss Albino mouse strains. However, the most frequent and detailed reports on the patency of the mPcomA are based on examinations of commonly utilized C57BL/6 mouse. For example, it has been estimated that only 1 in 10 C57BL/6 mice have an intact posterior circle of Willis (McColl et al. 2004).

The different nomenclature of the PCA and PcomA in mice compared to humans is not merely a semantic issue; it is important to note that if the above reports are correct, then this strongly suggests that the posterior circle of Willis in mice more closely resembles the human FTP configuration discussed above; and, in the absence of a patent mPcomA, this indicates that the majority of arterial supply to the PCA derives from the ICA. Indeed, the patency of the PcomA is correlated to the magnitude and variable severity of cerebral ischemia within and across several mouse strains in a number of experimental models including global forebrain ischemia models (Barone et al. 1993; Fujii et al. 1997; Yang et al. 1997; Kitagawa et al. 1998; Murakami et al. 1998; Beckmann et al. 1999; Wellons et al. 2000; Kelly et al. 2001; Yonekura et al. 2004; Tamaki et al. 2006; Cho et al. 2007; Zhen and Doré 2007), and focal middle cerebral artery occlusion models (Barone et al. 1993; Ozdemir et al. 1999; Beckmann et al. 1999; McColl et al. 2004; but see Connolly et al. 1996; Majid et al. 2000; Furuya et al. 2004).

From an interesting technical perspective, it worth noting that, as discussed above in rats, the pterygopalatine artery in mice appears to be an important source of extracranial collateral flow to the ICA; and this may also influence outcome following experimental cerebral ischemia (Tamaki et al. 2006; Chen et al. 2008). Further, differences in the vascular territories of the MCA and the
number of leptomeningeal anastomoses may also influence brain injury following experimental cerebral ischemia in mice (Maeda et al. 1998).

**Gerbils**

A similar situation is found in Mongolian Gerbils, a species in which the variability in outcome following cerebral ischemia is undoubtedly influenced by a consistent cerebral-vascular anomaly (Oostveen et al. 1992). In these animals the PcomA is consistently bilaterally absent. Importantly, the AcomA is also completely absent in ~20-30% of gerbils. However, strain and vendor-related differences in the patency of communicating channels and their influence on brain injury have been reported (Breuer and Mayevsky 1992; Laidley et al. 2005).

**Conclusions**

The above discussion serves to indicate that a number of differences in the circle of Willis exist in rodents. Furthermore, there is variability in the incidences of these anomalies even within a single strain. However, as a cautionary note, it should be noted that a comprehensive analysis of the cerebral-vasculature in mice has not been carried out. In the absence of large-scale studies with large numbers of mice it is difficult to draw conclusions on the incidences and frequencies of cerebral-vascular anomalies in potential collateral flow pathways; and this may be further confounded by seemingly broad strain-related variability. The lack of a standardized nomenclature and methods for examining and reporting the cerebral vasculature is also of concern, particularly when assessing and naming tiny vessels in small mammals such as mice. Any large scale characterization should ideally rely on both non-invasive imaging techniques in addition to post-mortem examinations, and should also incorporate a functional assessment of cerebral blood flow.

Additionally, interpreting the effects of vascular anomalies on brain injury following cerebral ischemia is further complicated by the use of multiple global and focal ischemic experimental models, which produce markedly different patterns and time-courses of brain injury. Furthermore, the variability in outcome following experimental cerebral ischemia may be related to a number of experimental factors including the site of the occlusion, failure to consider the geometry of the circle of Willis, technical skill, animal size, choice of surgical equipment, and failure to control for a number of systemic parameters (Hata et al. 1998; Tsuchiya et al. 2003;
Furuya et al. 2004; Connolly et al. 1996; Chen et al. 2008; Kuraoka et al. 2006; Howells et al. 2010). On a final note, it is worth mentioning that the ability of arteries to compensate for increased collateral flow also is influenced by the resistance of afferent and efferent vessels; thus, factors other than diameter or patency of communicating channels can potentially influence the extent of collateral flow during cerebral ischemia (Scremin 2004a). Still, the evidence for vascular anomalies in influencing post-ischemic brain injury is far more compelling in mice and gerbils than in rats.

**Appendix I. References**


Appendix II. Unilateral occlusion of the right common carotid artery does not affect EEG parameters in C57BL/6 mice

Hypoxia-ischemia (HI) is a two-step procedure that includes ligation of the right common carotid artery (RCCA) followed by respiratory hypoxia (8%O₂ for ~30 minutes). My studies and previous reports have demonstrated that HI results in extensive brain injury in the hemisphere ipsilateral to the RCCA ligation. The hypoxic episode is necessary because unilateral carotid artery ligation alone does not result in mortality or brain injury (Olson and McKeon 2004; Yoshizaki et al. 2008). This is presumably due to sufficient collateral cerebral blow flow from both the contralateral carotid artery and the basilar artery through the Circle of Willis. However, it has been demonstrated by others that unilateral common carotid artery occlusion can result in white matter lesions (Yoshizaki et al. 2008). Thus, despite the absence of extensive brain injury, RCCA ligation alone may result in subtle alterations in synaptic transmission.

Importantly, my results have indicated that HI can result in extensive suppression of ipsilateral hippocampal EEG activity; and that the suppression in ipsilateral EEG activity is good marker for assessing subsequent brain injury. In particular, EEG amplitude measurements and examinations of hippocampal theta rhythms have shown good predictive value. Thus, I sought to determine whether unilateral occlusion of the right common carotid artery can result in an alteration of ipsilateral or contralateral EEG activity in adult male C57BL/6 mice.

The experimental procedures are identical to that described previously. Mice were implanted with bilateral electrodes in the ipsilateral hippocampus and contralateral cortex (ipsilateral and contralateral refer to the RCCA ligation). Two groups of post-RCCA ligation mice were examined: at 1 hour (n=24 mice) and 1 week following RCCA ligation (n=14 mice). RCCA ligation alone did not result in mortality, motor seizures, or any obvious behavioral abnormalities. No significant differences in ipsilateral hippocampal EEG amplitude relative to baseline were noted at either 1 hour or 1 week post-RCCA ligation (p=0.149; one-way ANOVA; Figure 1A, left). Additionally, no significant differences in contralateral cortical EEG amplitude relative to baseline were noted at either 1 hour or 1 week post-RCCA ligation (p=0.531; one-way ANOVA; Figure 1A, right).
To further assess the possibility of synaptic deficits in the hippocampus, I examined hippocampal theta frequency and power at 1 week post-RCCA ligation. (Theta rhythms were not analyzed at 1 hour post-HI because animals were generally immobile at this time point, likely due to residual effects of the surgery or anesthesia.) No alterations in hippocampal theta frequency (p=0.577; paired t-test) or power (p=0.135; paired t-test) were observed relative to baseline at 1 week post-RCCA ligation (Figure 1B).

Collectively, these observations indicate that RCCA ligation alone does not result in appreciable alterations in EEG activity; and this therefore supports previous observations that have demonstrated that RCCA ligation alone does not result in extensive ipsilateral brain injury in C57BL/6 mice.

Appendix II. References


Appendix II. Figure 1. RCCA ligation alone does not affect EEG activity

A

ips. hipp amplitude (% of baseline)

125
100
75
50
25
0

(24)

1 hour post-RCCA occlusion

(14)

1 week post-RCCA occlusion

cont. cort amplitude (% of baseline)

125
100
75
50
25
0

(17)

1 hour post-RCCA occlusion

(10)

1 week post-RCCA occlusion

B

EEG frequency (Hz)

8
4
0

EEG amplitude (mV^2/Hz)

0.030
0.020
0.010
0

hipp theta
cort delta

baseline

1 week post RCCA occlusion

hipp theta
cort delta

1 hour post-RCCA occlusion
Appendix II. Figure 1. RCCA ligation alone does not affect EEG activity

A, Effects of right common carotid artery (RCCA) ligation alone on EEG amplitude. *Left*, ipsilateral hippocampal EEG amplitude was not significantly altered in the two groups of mice examined at 1 hour or 1 week post-RCCA ligation (p=0.149; One-way ANOVA). *Right*, contralateral cortical EEG amplitude was not significantly altered in the two groups of mice examined at 1 hour or 1 week post-RCCA ligation (p=0.531; One-way ANOVA). B, Effects of RCCA ligation on theta frequency and power. *Left*, ipsilateral hippocampal EEG theta frequency was not significantly altered in the two groups of mice examined at 1 week post-RCCA ligation (p=0.577; paired t-test). *Right*, ipsilateral hippocampal EEG theta power was not significantly altered in the two groups of mice examined at 1 week post-RCCA ligation (p=0.135; paired t-test).